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FORM PTO-1190 (REV 10-95)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				OMRF143 CIP2	
				U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
				08/765324	
INTERNATIONAL APPLICATION NO.		INTERNATIONAL FILING DATE		PRIORITY DATE CLAIMED	
PCT/US95/08331		30 June 1995		30 June 1994	
TITLE OF INVENTION					
Antibodies to Lipoproteins and Apolipoproteins and Methods of Use Thereof					
APPLICANT(S) FOR DO/EO/US					
KOREN, Eugen and KOSCEC, Mirna					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> has been transmitted by the International Bureau.</p> <p style="margin-left: 20px;">c. <input checked="" type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p style="margin-left: 20px;">a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p style="margin-left: 20px;">b. <input type="checkbox"/> have been transmitted by the International Bureau.</p> <p style="margin-left: 20px;">c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p style="margin-left: 20px;">d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. to 16. below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p style="margin-left: 20px;"><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: Copy of published application Verified Statement Claiming Small Entity Status</p>					

U.S. APPLICATION NO (if known, see 37 CFR 1.5)

INTERNATIONAL APPLICATION NO

PCT/US95/08331

ATTORNEY'S DOCKET NUMBER

OMR143 CIP2

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1)-(5)):

Search Report has been prepared by the EPO or JPO \$910.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)
..... \$700.00No international preliminary examination fee paid to USPTO (37 CFR 1.482)
but international search fee paid to USPTO (37 CFR 1.445(a)(2)) \$770.00Neither international preliminary examination fee (37 CFR 1.482) nor
international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$1040.00International preliminary examination fee paid to USPTO (37 CFR 1.482)
and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$ 910.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	44 - 20 =	24	X \$22.00
Independent claims	6 - 3 =	3	X \$80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ 260.00

\$ 528.00

\$ 240.00

\$

TOTAL OF ABOVE CALCULATIONS =

\$1,678.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement
must also be filed (Note 37 CFR 1.9, 1.27, 1.28).

\$ 839.00

SUBTOTAL =

\$ 839.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30
months from the earliest claimed priority date (37 CFR 1.492(f)).

\$

TOTAL NATIONAL FEE =

\$ 839.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property

+

\$ 40.00

TOTAL FEES ENCLOSED =

\$ 879.00

Amount to be:
refunded

charged

a. ☒ A check in the amount of \$ 879.00 to cover the above fees is enclosed.b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 01-2507. A duplicate copy of this sheet is enclosed.NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR
1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

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SIGNATURE

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31,284

REGISTRATION NUMBER

ANTIBODIES TO LIPOPROTEINS AND APOLIPOPROTEINS
AND METHODS OF USE THEREOF

Background of the Invention

This invention is generally in the field of methods and compositions for the determination and quantitation of lipoproteins and apolipoproteins in human blood.

10 Human plasma lipoproteins and apolipoproteins

Plasma lipoproteins are carriers of lipids from the sites of synthesis and absorption to the sites of storage and/or utilization. Lipoproteins are spherical particles with triglycerides and cholesterol esters in their core and a layer of phospholipids, nonesterified cholesterol and apolipoproteins on the surface. They are categorized into five major classes based on their hydrated density as very large, triglyceride-rich particles known as chylomicrons (less than 0.95 g/ml), very low density lipoproteins (VLDL, 0.95 to 1.006 g/ml), intermediate-density lipoproteins (IDL, 1.006 to 1.019 g/ml), low-density lipoproteins (LDL, 1.019 to 1.063 g/ml) and, high-density lipoproteins (HDL, 1.063 to 1.210 g/ml). Plasma lipoproteins can be also classified on the basis of their electrophoretic mobility. HDL co-migrate with α -globulins, LDL with β -globulins, VLDL between α - and β -globulins with so called pre- β globulins, whereas chylomicrons remain at the point of application. (Osborne, J.D. and Brewer, B. Jr. Adv. Prot. Chem. 31:253-337 (1977); Smith, L.C. et al. Ann. Rev. Biochem., 47:751-777 (1978)).

Apolipoproteins are protein components of lipoproteins with three major functions: (1) maintaining the stability of lipoprotein particles, (2) acting as cofactors for enzymes that act on lipoproteins, and (3) removing lipoproteins from

circulation by receptor-mediated mechanisms. The four groups of apolipoproteins are apolipoproteins A (Apo A), B (Apo B), C (Apo C) and E (Apo E). Each of the three groups A, B and C consists of two or more distinct proteins. These are for Apo A: Apo A-I, Apo A-II, and Apo A-IV, for Apo B: Apo B-100 and Apo B-48; and for Apo C: Apo C-I, Apo C-II and Apo C-III. Apo E includes several isoforms.

Each class of lipoproteins includes a variety of apolipoproteins in differing proportions with the exception of LDL, which contains Apo B-100 as a sole apolipoprotein. Apo A-I and Apo A-II constitute approximately 90 percent of the protein moiety of HDL whereas Apo C and Apo E are present in various proportions in chylomicrons, VLDL, IDL and HDL. Apo B-100 is present in LDL, VLDL and IDL. Apo B-48 resides only in chylomicrons and so called chylomicron remnants (Kane, J.P., Method. Enzymol. 129:123-129 (1986)).

Lipoprotein metabolism is a very complex process involving exogenous and endogenous pathways as well as a reverse cholesterol transport. In the exogenous pathway, the triglycerides and cholesterol from an individual's diet are incorporated into chylomicrons which enter into the blood stream via intestinal lymph. Lipoprotein lipase hydrolyzes the triglyceride component of chylomicrons into free fatty acids which are taken up by muscle cells and/or adipocytes. As the triglyceride core of chylomicrons is depleted, chylomicron remnant particles are formed and removed from the circulation via chylomicron remnant receptor present on the surface of hepatic cells.

In the endogenous pathway, the liver synthesizes triglycerides and cholesterol. The endogenously made triglycerides and cholesterol are

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packed into triglyceride rich VLDL particles and
secreted into the circulation. Once in the blood,
most of the triglyceride content of VLDL particles
is hydrolyzed by lipoprotein lipase, releasing free
5 fatty acids to be used as a source of energy or for
storage. As a result of this process, VLDL parti-
cles diminish in size and increase in density and
are converted into VLDL remnants or IDL. Further
processing includes additional lipolysis and
10 exchange of lipids and apolipoproteins between IDL
and HDL, leading to the formation of LDL which
contain mostly cholesterol esters in the core and
phospholipids and Apo B-100 on the surface. LDL
particles are taken up by the hepatic and
15 extrahepatic cells via specific LDL-receptor.

The reverse cholesterol transport pathway
starts with the secretion of nascent HDL particles
which are produced by the liver and intestine.
These disk-like particles consist primarily of
20 phospholipids surrounded by Apo A-I. They accept
free cholesterol from peripheral tissues which is
esterified and translocated into the core of HDL
particles, which become spherical and ready to
deliver their cholesterol content to hepatocytes.
25 During the degradation of VLDL and LDL, HDL
particles also accept free cholesterol and
apolipoproteins from these lipoproteins.

Role of lipoproteins in atherosclerosis

Atherosclerosis is a chronic disease
30 characterized by progressive deposition of
cholesterol, fibrous elements and minerals in
arterial walls. Atherosclerosis is the underlying
pathophysiological process of coronary heart
disease (CHD), one of the leading causes of death
35 in Western World (Report of the Working Group on
Atherosclerosis of the National Heart and Lung and
Blood Institute, 2 (Washington, D.C.: Government

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Printing Office, 1981) DHEW Publication No. (NIH) 82-2035). Although development of CHD is a very complex process influenced by many contributing factors, subintimal cholesterol deposition in coronary arteries is one of the earliest and most important events during the course of disease. The major source of cholesterol found in arterial wall deposits is plasma lipoproteins. Because of their diverse metabolic roles and properties, lipoproteins associate differently with the risk of developing CHD.

LDL particles constitute approximately two-thirds of total cholesterol (TC) and form the primary atherogenic fraction of the serum cholesterol. Many epidemiological and clinical studies have shown that increased LDL levels in the blood are associated with an increased risk of CHD. For example, the results of the Lipid Research Clinics trial have shown that reduction of LDL-cholesterol (LDL-C) is associated with a significant decrease in CHD incidence (The Lipid Research Clinics Coronary Primary Prevention Trial results:II. JAMA 251:365-374. (1984)).

The evidence relating CHD and triglyceride-rich lipoproteins such as VLDL is not as strong as for the LDL. Many studies have shown a positive correlation between elevated serum triglyceride levels and increased risk of CHD. However, the independent link between elevated serum triglyceride (TG) and CHD breaks down when multivariate analyses are used to control statistically for the effects of total cholesterol (TC) and HDL-cholesterol (HDL-C). These observations suggest that increased CHD risk noted in patients with hypertriglyceridemia could be due to either the accumulation of triglyceride-rich particles that are uniquely atherogenic in some people or to

the association with reduced HDL-C (Assmann, G. et al., Am. J. Cardiol., 68:1-3 (1991)). Remnants of triglyceride-rich particles, (for example, chylomicron and VLDL remnants) which are found in IDL are also atherogenic (Krauss, R.M., Am. Heart J., 112:578-582 (1987)).

In contrast to the atherogenic potential of LDL, VLDL and VLDL remnants, HDL are inversely correlated with CHD, so that individuals with low concentrations of HDL-C have an increased incidence of CHD (Gordon, T. et al., Am. J. Med., 62:707-714 (1977); Miller, N.E. et al., Lancet, 1:965-968 (1977); Miller, G. J. and Miller, N.E., Lancet, 1:16-19 (1975)). At the other extreme, individuals with high concentrations of HDL, such as found in familial hyperalphalipoproteinemia, seldom express symptoms of CHD. The fact that pre-menopausal females have higher HDL concentrations and less CHD compared to males, also supports the anti-atherogenic role of HDL. Furthermore, postmenopausal women have a significant increase in CHD risk while their HDL concentrations decrease.

Measurement of LDL

LDL consists of a hydrophobic lipid core composed of cholesterol esters and triglycerides. The lipid core of the LDL particle is surrounded by an amphipathic coat composed of phospholipids, unesterified cholesterol and Apo B. Each LDL particle contains one molecule of Apo B-100. On a weight basis, LDL is composed of 38 percent cholesterol ester, 22 percent phospholipid, 21 percent protein, 11 percent triglyceride and 8 percent unesterified cholesterol.

Accurate measurements of LDL using presently available technology depends on separation of LDL particles from other lipoproteins. Once the LDL particles are separated, their concentration can be

Several ultracentrifugation methods have been developed over the years to separate serum lipoproteins. Analytical ultracentrifugation was developed in the 1950s and continues to be used today in some research laboratories. In this technique, lipoproteins are separated by analytical ultra-centrifugation and quantitated by optical refraction. This method of quantitation measures lipoprotein mass, but does not give any information about lipid or protein composition. Sequential ultracentrifugation was developed in 1955 to overcome some of the limitations of analytical ultracentrifugation. In this technique, lipoproteins are separated by repeated ultracentrifugations after progressively increasing the sample density. Lipoproteins can be isolated within any desired density interval and in sufficient quantities to allow for multiple chemical analyses. Sequential ultracentrifugation continues to be used today for preparative isolation of lipoproteins. However, the ultracentrifugation methods are too expensive and time consuming for the purpose of measuring LDL-C levels to assess lipoprotein abnormalities and CHD risk in routine clinical application. Other methods for separating LDL include size-exclusion and other types of chromatography, electrophoresis, and precipitation. The size-exclusion chromatography methods include agarose column chromatography and high-performance gel filtration column chromatography. The time required for analysis, typically 24 hours, is the major difficulty with agarose column chromatography. The development of high-performance liquid chromatography (HPLC) methods has reduced the

analysis time, but has increased the cost and complexity of the procedure. Affinity chromatography using anti-LDL antibodies, heparin, or dextran sulfate linked to SEPHAROSE™ (Pharmacia LKB, Piscataway, NJ) gels has also been used to isolate LDL.

Electrophoresis methods, which separate lipoproteins according to their charge in addition to size, have been used in many clinical laboratories. This technique is helpful in qualitative assessment of various types of hyperlipoproteinemias. Agarose gel electrophoresis at pH 8.6, followed by visualization using lipophilic stains such as Oil Red O, Sudan Black B or Sudan Red 7B, have been commonly used with commercial reagents packaged as kits, for example, as sold by Ciba Corning (Medfield, MA) Lipoprotein concentrations are then estimated by densitometry based on the color intensity of the separated bands.

Several methods for selective chemical precipitation of LDL have been described and commercialized (Mulder, K. et al., Clin. Chim. Acta 143:29-35 (1984)). The precipitation methods, which quantitate LDL-C as the difference between the total cholesterol and the sum of VLDL- and HDL-cholesterol (Friedewald, W.T. et al., Clin. Chem. 18:499-502 (1972)), are precise and produce reasonably accurate results relative to ultracentrifugation methods when serum TG values are low. However, most investigators have found that the precipitation methods are plagued with systematic errors when samples with high TG levels are analyzed.

Most recently, a method was developed which uses latex beads coated with affinity-purified polyclonal goat antisera directed against

apolipoproteins in HDL and VLDL (Sigma, St. Louis, MO). In this method, a plasma or serum sample is incubated with the beads for 5 to 10 minutes at room temperature and then centrifuged for 5 minutes to remove the HDL and VLDL bound to the beads. The remainder of the sample is then assayed for cholesterol using a standard enzymatic cholesterol assay (Sigma St. Louis, MO) to obtain a value for the LDL-C, the presumed remaining source of cholesterol in the sample.

Techniques used for measurement of LDL by its Apo B content include radioimmunoassay; enzyme immunoassay (ELISA competitive or capture systems), fluorescence immunoassay, radial immunodiffusion, nephelometry, turbidimetry and electroimmunoassay.

The National Cholesterol Education Program (NCEP) recommended the determination of LDL-C concentration in diagnosis and treatment of hypercholesterolemia. According to NCEP, concentrations lower than 130 mg/dl in adults are considered desirable, concentrations between 130 and 150 mg/dl are borderline high, and concentrations above 160 md/dl are high (see Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults, Arch. Intern. Med. 148:36-69 (1988)). The NCEP also recommended determining LDL-cholesterol concentrations for children and adolescents since the high LDL correlates with the extent of coronary and aortic atherosclerosis in this age group as well as development of CHD later in life. Cholesterol values of 110 mg/dl are desirable, values between 110 and 129 mg/dl are borderline high, and values above 130 mg/dl are considered high in children and adolescents (see Report of the Expert Panel on

Blood Cholesterol Levels in Children and Adolescents, Pediatrics 89:525-584 (1992)).

Measurement of HDL

5 HDL, the smallest in size of the lipoproteins, includes a family of lipoprotein particles that exist in a constant state of dynamic flux as they interact with LDL, IDL and VLDL. HDL have the highest proportion of protein (50 percent) relative to lipid compared to other lipoproteins. The major
10 HDL proteins are Apo A-I and Apo A-II, with lower concentrations of Apo C(I,II & III), E, and A-IV. Phospholipids are the principal lipid component of HDL, with cholesterol esters, unesterified cholesterol, and TG present in lower
15 concentrations.

As in the case of LDL, HDL is typically measured after its separation from other lipoproteins and quantification of cholesterol in the HDL (HDL-C). As described above, the
20 separation of HDL can be accomplished by ultracentrifugation, chromatographic procedures, electrophoresis and precipitation. The reliability of lipoprotein quantitations following separation by ultracentrifugation techniques depends upon both
25 the performance of the analytical quantitation method, such as cholesterol analysis, and the skills of the technologist in performing accurate recovery and transfer of the lipoprotein fractions from the ultracentrifuge tube. HDL-C is more
30 easily quantitated by selective precipitation techniques compared to either ultracentrifugation or electrophoretic methods. Currently, the majority of clinical laboratories use either dextran sulfate or sodium phosphotungstate procedures for HDL-C
35 analysis (Warnick, G.R. et al., Clin. Chem. 28:1379-1388 (1982); Lopes-Virella, M.F. et al., Clin. Chem. 23:882-884 (1977)). According to NECP

guidelines, patients with HDL-C levels below 35 mg/dl are considered to be at risk for CHD.

LDL/HDL Ratio

Some studies have demonstrated that the ratio
5 between LDL-C and HDL-C represents a better
predictor of CHD than either of these two
parameters alone (Arntzenius, A.C., Acta. Cardiol.,
46:431-438 (1991); Barth J.D. and Arntzenius, A.C.,
Eur. Heart J., 12:952-957 (1991); Ortola, J. et
10 al., Clin. Chem., 38:56-59 (1992); Gohlke H., Wien
Klin. Wochenschr., 104:309-313 (1992)).

LPA-I and LPA-I:A-II Lipoprotein Particles

There are two subpopulations of HDL
lipoprotein particles known as LPA-I and LPA-I:A-II
15 (Koren, E. et al. Clin. Chem., 33:38-43 (1987)).
LPA-I particles contain Apo A-I but no Apo A-II
while LPA-I:A-II particles contain both
apolipoproteins. These HDL subpopulations can be
measured by enzyme immunoassay (Koren, E. et al.
20 Clin. Chem., 33:38-43 (1987)) or electroimmunoassay
(Atmeh, R.F. et al., Biochim. Biophys. Acta,
751:175-188 (1983)). Their importance has been
emphasized by several studies which demonstrated
that LPA-I is a more active component in reverse
25 cholesterol transport and, therefore, more anti-
atherogenic than other lipoproteins (Puchois, P. et
al., Atherosclerosis, 68:35-40 (1987); Fruchart,
J.C. and Ailhaud, G., Clin. Chem., 38:793-797
(1992)).

Measurements of VLDL, IDL, C-III and E ratios

Triglyceride-rich VLDL as well as their
remnants (IDL) can be separated by the above
ultracentrifugational, chromatographic and
electrophoretic methods and quantified by
35 determination of their cholesterol content.
Although atherogenic, these lipoprotein particles
are not commonly measured in routine clinical

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laboratories. Instead, serum triglyceride concentration in the fasting state is considered representative of the VLDL content and is used traditionally in the assessment of the VLDL-related CHD risk. More recently, measurements of the so-called C-III and E ratios have been proposed as reliable predictors of the VLDL-related CHD risk. The principle of these measurements is to precipitate all Apo B-100-containing particles (VLDL, IDL and LDL) with heparin which leaves HDL in the heparin supernate. This separation is followed by an immunochemical determination of Apo C-III or Apo E in the heparin precipitate and heparin supernate and calculation of the corresponding ratios by dividing C-III or E concentration in heparin supernate with their respective concentrations in heparin precipitate (Alaupovic, P., Can. J. Biochem., 59:565-579 (1981)). Most of the Apo C-III and Apo E in the heparin precipitate is associated with Apo B in VLDL and VLDL remnant (IDL) particles. The C-III and E in the heparin supernate is associated with Apo A-I in HDL particles. Apo C-III and/or Apo E in the heparin precipitate reflects the concentration of VLDL and VLDL-remnant particles both of which are atherogenic. The Apo C-III and Apo E in the heparin supernate represents HDL particles which are anti-atherogenic. Therefore, a low C-III and E ratio is associated with increased risk of CHD because it reflects either high VLDL and IDL and normal HDL or, more frequently, high VLDL and IDL combined with low HDL. In fact, the predictive power of C-III ratio has surpassed that of triglycerides in several clinical studies (Alaupovic, P. and Blankenhorn, D.H., Klin. Wochenschr., 60:38-40 (1990); Blankenhorn, D.H. et al., Circulation 81:470-478 (1990)).

Measurements of Apo A-I and B

Apo B-100 is an integral component of the four major atherogenic lipoproteins: VLDL, IDL, LDL and Lp(a). Apo B-100 is distinguished from Apo B-48, which is found only in lipoproteins of intestinal origin, such as chylomicrons and chylomicron remnants. Apo B-48 is usually undetectable in the systemic circulation, except in rare subjects with Type I, III, or V hyperlipidemia. Apo B's initial function in VLDL and IDL appears to be structural; however, with exposure of binding domains on LDL, it becomes responsible for interaction with high-affinity LDL receptors on cell surfaces, which results in uptake and removal of LDL from the circulation. Several studies have shown that an increased Apo B level in blood is a reliable marker for coronary atherosclerosis (Sniderman, A. et al., Proc. Natl. Acad. Sci. USA, 77:604-608 (1980); Kwiterovich, P.O. et al., Am. J. Cardiol., 71:631-639 (1993); McGill et al. Coron. Artery Dis., 4:261-270 (1993); Tornvall, P. et al., Circulation, 88:2180-2189 (1993)).

Apo A-I is the major protein constituent of lipoproteins in the high density range. Apo A-I may also be the ligand that binds to a proposed hepatic receptor for HDL removal. A number of studies support the clinical sensitivity and specificity of Apo A-I as a negative risk factor for atherosclerosis (Avogaro, P. et al., Lancet, 1:901-903 (1979); Maciejko, J.J. et al., N. Engl. J. Med., 309:385-389 (1983)). Some investigators have also described Apo A-I/Apo B ratio as a useful index of atherosclerotic risk (Kwiterovich, P.O. et al., Am. J. Cardiol., 69:1015-1021 (1992); Kuyl, J.M. and Mendelsohn, D., Clin. Biochem., 25:313-316 (1992)).

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Techniques used for both Apo A-I and B are confined to immunological procedures using antibodies directed against Apo A-I or B and include radio-immunoassay (RIA), enzyme immunoassay (ELISA), competitive or capture systems, fluorescence immunoassay, radial immunodiffusion, nephelometry, turbidimetry and electroimmunoassay.

To summarize, there are several lipoprotein related parameters that are currently used as predictors of CHD. Some of them represent atherogenic lipoproteins (total cholesterol, triglycerides, LDL, IDL, VLDL, Lp(a) and Apo B and are positively associated with CHD whereas the others are anti-atherogenic factors, HDL, Apo A-I and LPA-I which are inversely related to the disease. The ratios of some of these parameters, such as LDL/HDL, Apo A-I/Apo B, C-III and E ratio, appear to be even more sensitive predictors of CHD because each of them reflects both anti-atherogenic and atherogenic factors in a single parameter.

All of the methods currently used to determine lipoprotein related risk factors require a laboratory with the necessary equipment and trained personnel to carry out each of the technical steps, to perform the necessary calculations and to interpret the results. The only exception is a new total cholesterol measurement device (AccuMeter Cholesterol Self-Test) developed by ChemTrack (Sunnyvale, CA) and designed for home use. However, a total cholesterol level is a less sensitive predictor compared to the levels of specific lipoproteins, apolipoproteins or ratios thereof.

It is therefore an object of the present invention to provide methods and means to rapidly and reliably determine levels of specific lipoproteins, apolipoproteins or the ratios thereof

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in whole blood, serum or plasma without the necessity of laboratory equipment or technically trained personnel.

It is another object of the present invention
5 to provide antibodies immunoreactive with specific epitopes on lipoproteins, such as those on LDL, VLDL and HDL, that enable rapid and reliable determinations of levels of lipoproteins and/or apolipoproteins in whole blood, serum or plasma.

10

Summary of the Invention

Compositions and methods using antibodies which are immunoreactive with specific
15 apolipoproteins to determine the concentrations of lipoproteins such as HDL and LDL, and/or apolipoproteins in human blood, serum or plasma sample, are described. Monoclonal antibodies (MAbs) are described that specifically bind to
20 epitopes present in apolipoproteins and lipoproteins, enabling rapid and reliable determinations of levels of specific blood lipoprotein and/or apolipoprotein levels, including Apo B-100, Apo A-I, Apo A-II, Apo C-III, and Apo E, and
25 thereby determination of relative ratios of HDL and LDL and Lp(a) and Lp(aII).

In a preferred embodiment, the compositions are strips of a solid phase material coated with one or more of the antibodies and are referred to
30 herein as "dipsticks". The dipsticks specifically bind a lipoprotein or apolipoprotein when dipped into a protein sample. The amount of lipid associated with a bound lipoprotein or the amount of apolipoprotein bound on the dipstick is
35 quantitated using an appropriate method, for example, by staining with a lipid stain or reaction with a second labelled antibody. The intensity of

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the stain on the dipstick is proportional to the concentration of the lipoprotein lipid or apolipoprotein circulating in the blood and can be quantitated by comparison with standards containing
5 known amounts of lipid. The dipsticks can be provided alone or in kits which enable the lay person to carry out the assay without the need of a physician or technical laboratory.

The MAbs, whether immobilized or in solution,
10 can be used not only as components of dipsticks, but also in a variety of other methods, including enzyme immunoassays, radioimmunoassays as well as fluorescent and chemiluminescent immunoassays to determine lipoproteins and apolipoproteins in
15 biological samples, and in purification of the apolipoprotein or lipoprotein with which they are immunoreactive.

Detailed Description of the Invention

20 Methods and compositions to determine the concentration of specific lipoproteins and/or apolipoproteins, such as LDL and HDL, which when present at elevated levels in the body are causally
25 related to an increased or decreased risk of CHD have been developed. In a preferred embodiment, blood lipoprotein and/or apolipoprotein molecules in a patient sample are bound to specific antibodies immobilized on specially prepared strips
30 of solid phase material and to the bound lipoprotein and/or apolipoproteins visualized using specific colored staining reagents. The intensity of the color is proportional to the concentration of the lipid component or apolipoprotein component
35 of the lipo-protein circulating in the blood. In a second preferred embodiment, the blood lipoprotein and/or apolipoprotein molecules in a patient sample

are immunoprecipitated by reaction with specific MABs lipoproteins and/or apolipoproteins, such as LDL and HDL.

I. Antibodies to Lipoproteins and Apolipoproteins

5 A. MAb methodology

Monoclonal antibody technology can be used to obtain MABs useful in methods to rapidly and reliably determine blood lipoproteins and apolipoproteins (Galfré, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981) incorporated herein by reference). Briefly, hybridomas are produced using spleen cells from mice immunized with a particular apolipoprotein. The spleen cells of each immunized mouse is fused with mouse myeloma Sp 2/0 cells, for example using the polyethylene glycol fusion method of Galfré, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981). Growth of hybridomas, selection in HAT medium, cloning and screening of clones against antigens are carried out using standard methodology (Galfré, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981)).

HAT-selected clones are injected into mice to produce large quantities of MAB in ascites as described by Galfré, G. and Milstein, C., Methods Enzymol., 73:3-46 (1981), which can be purified using protein A column chromatography (BioRad, Hercules, CA). MABs are selected on the basis of their (a) specificity for a particular apolipoprotein, (b) high binding affinity, (c) isotype, and (d) stability.

30 B. Testing for specificity and affinity

MABs can be screened or tested for specificity using any of a variety of standard techniques, including Western Blotting (Koren, E. et al., Biochim. Biophys. Acta 876:91-100 (1986)) and enzyme-linked immunosorbent assay (ELISA) (Koren, E. et al., Biochim. Biophys. Acta 876:91-100

(1986)), as described in more detail in the following examples.

In ELISA, separate wells in microtiter plates are coated with purified apolipoproteins which adsorb to the wall of the wells. The wells are then treated with a blocking agent, such as bovine serum albumin or nonfat milk proteins, to cover areas in the wells not bound by antigen. Ascites fluid or other antibody-containing preparation can then be applied to each well in varying concentrations and adequate time allowed for MAb to bind the antigen adsorbed on the wall of each well. The presence of MAb bound to antigen in a well can then be detected using a standard enzyme-conjugated anti-mouse antibody which will bind MAb that has bound to apolipoprotein in the well. Wells in which MAb is bound to antigen are then identified by adding a chromogenic substrate for the enzyme conjugated to the anti-mouse antibody and color production detected by an optical device such as an ELISA plate reader.

MAbs that bind to a single apolipoprotein with no significant detectable crossreactivity with other apolipoproteins are considered specific. To determine specificity of MAbs for a particular lipoprotein, individual wells on ELISA plates are coated with purified chylomicrons VLDL, LDL and HDL and subjected to the identical procedure. To determine whether or not two MAbs specific for the same apolipoprotein bind to different epitopes, a competitive ELISA is performed. For example, one of the MAbs is biotinylated. Mixtures containing a constant concentration of the biotinylated MAb and increasing concentrations of the nonbiotinylated MAb are incubated with wells coated with the apolipoprotein or lipoprotein antigen. Quantity of biotinylated antibody bound to the coated antigen

is determined using a streptavidin-peroxidase conjugate and a chromogenic substrate. Decreased binding of the biotinylated MAb with increasing concentrations of the nonbiotinylated MAb indicates that the two MAbs compete for the same epitope. If the biotinylated MAb binds equally to the antigen as does the unlabelled MAb despite increasing concentrations of the nonbiotinylated MAb, the two antibodies do not compete for the same epitope. This competition can be complete or partial. Affinity of MAbs can be determined using radioactively labelled (^{125}I) lipoproteins or apolipoproteins and purified MAbs as described by Koren, E. et al., Biochim. Biophys. Acta 876:91-100 (1986), incorporated herein by reference).

C. Proteolytic cleavage of antibodies

It may be desirable to produce and use functional fragments of an MAb for a particular application. The well-known basic structure of a typical IgG molecule is a symmetrical tetrameric Y-shaped molecule of approximately 150,000 to 200,000 daltons consisting of two identical light polypeptide chains (containing about 220 amino acids) and two identical heavy polypeptide chains (containing about 440 amino acids). Heavy chains are linked to one another through at least one disulfide bond. Each light chain is linked to a contiguous heavy chain by a disulfide linkage. An antigen-binding site or domain is located in each arm of the Y-shaped antibody molecule and is formed between the amino terminal regions of each pair of disulfide linked light and heavy chains. These amino terminal regions of the light and heavy chains consist of approximately their first 110 amino terminal amino acids and are known as the variable regions of the light and heavy chains. In addition, within the variable regions of the light

and heavy chains there are hypervariable regions which contain stretches of amino acid sequences, known as complementarity determining regions (CDRs). CDRs are responsible for the antibody's specificity for one particular site on an antigen molecule called an epitope. Thus, the typical IgG molecule is divalent in that it can bind two antigen molecules because each antigen-binding site is able to bind the specific epitope of each antigen molecule. The carboxy terminal regions of light and heavy chains are similar or identical to those of other antibody molecules and are called constant regions. The amino acid sequence of the constant region of the heavy chains of a particular antibody defines what class of antibody it is, for example, IgG, IgD, IgE, IgA or IgM. Some classes of antibodies contain two or more identical antibodies associated with each other in multivalent antigen-binding arrangements.

Proteolytic cleavage of a typical IgG molecule with papain is known to produce two separate antigen binding fragments called Fab fragments which contain an intact light chain linked to an amino terminal portion of the contiguous heavy chain via by disulfide linkage. The remaining portion of the papain-digested immunoglobulin molecule is known as the Fc fragment and consists of the carboxy terminal portions of the antibody left intact and linked together via disulfide bonds. If an antibody is digested with pepsin, a fragment known as an F(ab')₂ fragment is produced which lacks the Fc region but contains both antigen-binding domains held together by disulfide bonds between contiguous light and heavy chains (as Fab fragments) and also disulfide linkages between the remaining portions of the contiguous heavy chains (Handbook of Experimental Immunology, Vol 1:

Immunochemistry, Weir, D.M., Editor, Blackwell Scientific Publications, Oxford (1986)).

Fab and F(ab')₂ fragments of MAbs that bind particular blood apolipoproteins or lipoproteins can be used in place of whole MAbs in methods for detecting or quantifying such blood proteins or the lipids associated with such proteins. Because Fab and F(ab')₂ fragments are smaller than intact antibody molecules, more antigen-binding domains can be immobilized per unit area of a solid support than when whole antibody molecules are used. As explained below, rapid, easy and reliable assay systems can be made in which antibodies or antibody fragment that specifically bind apolipoproteins and lipoproteins are immobilized on solid phase materials.

D. Recombinant antibodies

Recombinant DNA methods have been developed which permit the production and selection of recombinant antibodies which are single chain antigen-binding polypeptides known as single chain Fv fragments (ScFvs or ScFv antibodies). ScFvs bind a specific epitope of interest and can be produced using any of a variety of recombinant bacterial phage-based methods, for example as described in Lowman, H.B. et al., Biochemistry, 30: 10832-10838 (1991); Clackson, T. et al., Nature, 352: 624-628 (1991); and Cwirla, S.E. et al., Proc. Natl. Acad. Sci. USA, 87: 6378-6382 (1990), incorporated herein by reference. These methods are usually based on producing genetically altered filamentous phage, such as recombinant M13 or fd phages, which display on the surface of the phage particle a recombinant fusion protein containing the antigen-binding ScFv antibody as the amino terminal region of the fusion protein and the minor phage coat protein g3p as the carboxy terminal

region of the fusion protein. Such recombinant phages can be readily grown and isolated using well-known phage methods. Furthermore, the intact phage particles can usually be screened directly for the presence (display) of an antigen-binding ScFv on their surface without the necessity of isolating the ScFv away from the phage particle.

To produce an ScFv, standard reverse transcriptase protocols are used to first produce cDNA from mRNA isolated from a hybridoma that produces an MAb for an antigen of interest. The cDNA molecules encoding the variable regions of the heavy and light chains of the MAb can then be amplified by standard polymerase chain reaction (PCR) methodology using a set of primers for mouse immunoglobulin heavy and light variable regions (Clackson, T. et al., Nature, 352:624-628 (1991), incorporated herein by reference). The amplified cDNAs encoding MAb heavy and light chain variable regions are then linked together with a linker oligonucleotide in order to generate a recombinant ScFv DNA molecule. The ScFv DNA is ligated into a filamentous phage plasmid designed to fuse the amplified cDNA sequences into the 5' region of the phage gene encoding the minor coat protein called g3p. *Escherichia coli* bacterial cells are then transformed with the recombinant phage plasmids, and filamentous phage grown and harvested. The desired recombinant phages display antigen-binding domains fused to the amino terminal region of the minor coat protein. Such "display phages" can then be passed over immobilized antigen, for example, using the method known as "panning", see Parmley, S.F. and Smith, G.P., Adv. Exp. Med. Biol., 251:215-218 (1989); Cwirla, S.E. et al., Proc. Natl. Acad. Sci. USA, 87: 6378-6382 (1990), incorporated herein by reference, to adsorb those

phage particles containing ScFv antibody proteins that are capable of binding antigen. The antigen-binding phage particles can then be amplified by standard phage infection methods, and the amplified recombinant phage population again selected for antigen-binding ability. Such successive rounds of selection for antigen-binding ability, followed by amplification, select for enhanced antigen-binding ability in the ScFvs displayed on recombinant phages. Selection for increased antigen-binding ability may be made by adjusting the conditions under which binding takes place to require a tighter binding activity. Another method to select for enhanced antigen-binding activity is to alter nucleotide sequences within the cDNA encoding the binding domain of the ScFv and subject recombinant phage populations to successive rounds of selection for antigen-binding activity and amplification (see, Lowman, H.B. et al., Biochemistry, 30: 10832-10838 (1991) and Cwirla, S.E. et al., Proc. Natl. Acad. Sci. USA, 87: 6378-6382 (1990)).

Once an ScFv is selected, the recombinant antibody can be produced in a free form using an appropriate vector in conjunction with *E. coli* strain HB2151. These bacteria actually secrete ScFv in a soluble form, free of phage components (Hoogenboom H.R. et al., Nucl. Acids Res., 19:4133-4137 (1991), incorporated herein by reference). The purification of soluble ScFv from the HB2151 bacteria culture medium can be accomplished by affinity chromatography using antigen molecules immobilized on a solid support such as AFFIGEL™ (BioRad, Hercules, CA).

More recent developments in the recombinant antibody technology demonstrate possibilities for further improvements such as increased avidity of binding by polymerization of ScFvs into dimers and

tetramers (Holliger, P. et al., Proc. Natl. Acad. Sci. USA, 90: 6444-6448 (1993); Mezes, P. Construction and Biodistribution Studies of Multivalent Single-Chain Antibodies, The Fourth Annual IBC International Conference on Antibody Engineering, December 1993, Coronado, CA,; Ito, W. and Kurosawa, Y., J. Biol. Chem., 268: 20668-20675 (1993), incorporated herein by reference).

Because ScFvs are even smaller molecules than Fab or F(ab')₂ fragments, they can be used to attain even higher densities of antigen binding sites per unit of surface area when immobilized on a solid support material than possible using whole antibodies, F(ab')₂, or Fab fragments. Furthermore, recombinant antibody technology offers a more stable genetic source of antibodies, as compared with hybridomas. Recombinant antibodies can also be produced more quickly and economically using standard bacterial phage production methods.

As demonstrated below, the availability of hybridomas which produce MAbs to Apo B-100, Apo A-I, Apo A-II, Apo C-III, and Apo E enables the production of recombinant antibodies to these same antigens.

E. Anti-Apo Monoclonal antibodies (MAbs)

Unless specifically stated otherwise, the term "MAbs" includes natural and recombinant antibodies and fragments thereof.

MAbs to apolipoprotein (Apo) A-I, A-II, B, C-III and E can be used either alone or in various combinations to obtain a useful determination of the body's circulating levels of lipoproteins and/or apolipoproteins. MAbs used for making dipsticks, such as HB₃CB₃, D₆, AIbD₅, and CdB₅, described below, possess very high affinity constants ranging from 10⁸ to 10¹² M⁻¹ as determined by the methods described by Koren, et al., Biochim.

Biophys. Acta, 876:91-100 (1986); Biochim. Biophys. Acta, 876:101-107 (1986), incorporated herein by reference. An antibody coating a solid phase material is expected to bind a sufficient quantity of lipoprotein within a relatively short period of time (approximately two to five minutes), and to retain the captured lipoprotein during subsequent washing and staining for bound lipoprotein. It should be understood that while the descriptions below are the best antibodies presently known for making the compositions described herein, the methods described or incorporated by reference herein by citation to prior publications can be used by those skilled in the art to make other suitable antibodies having similar affinity and specificity which are functionally equivalent to those used in the following examples.

Monoclonal antibodies (MAbs) to apolipoproteins A-I, A-II, C-III and E were produced by immunization of Balb/c mice (Jackson Laboratories, St. Louis, MO) with purified apolipoproteins. All apolipoproteins were purified using well-established methods (Curry, M.D. et al., Clin. Chem. 22:315-322 (1976); Curry, M.D. et al., Clin. Chem. 24:280-286 (1978); Curry, M.D. et al., Biochim. Biophys. Acta 439:413-425 (1976); and Curry, M.D. et al., Biochim. Biophys. Acta 617:503-513 (1980)).

From a library of several hundred MAbs, two antibodies directed against Apo A-I, one against Apo A-II, two against Apo B, one against Apo C-III and two against Apo E were selected for the methods and compositions described herein. The MAbs were selected on the basis of their (a) specificity, (b) high binding affinity, (c) isotype (class of antibody), and (d) stability under the conditions described below. Using commercially available

isotype specific anti-mouse antibodies (Kirkegaard and Perry Laboratory, Gaithersburg, MD) all of the MAbs were shown to belong to the IgG1 class and possess kappa light chains.

5 Antibodies to Apo B

Two MAbs specific for Apo B, D₆ and HB₃CB₃ MAbs, were developed and found to be useful for the methods and compositions described below. D₆ and HB₃CB₃ MAbs bind to sterically distant epitopes on
10 Apo B.

Antibodies to Pan B

D₆ MAb is an antibody with equal binding and high affinity for all Apo B-containing lipoproteins in human plasma, as described by Koren, E. et al.,
15 Biochim. Biophys. Acta, 876:91-100 (1986); Koren, E. et al., Biochim. Biophys. Acta, 876:101-107 (1986), specifically including Apo B-48 and Apo B-100. D₆ binds to an epitope localized at the amino terminal half of Apo B and recognizes both B-48 and
20 B-100.

D₆ was produced after immunization of mice with a narrow cut of low density (1.021 to 1.006 g/ml) lipoproteins (LDL) containing apolipoprotein B (Apo B) as a sole protein (Smith, L.C. et al., Ann. Rev. Biochem., 47: 751-777 (1978)). Hybridomas were
25 produced using spleen cells from immunized mice. The fusion of spleen cells with mouse myeloma Sp 2/0 cells was carried out using the polyethylene glycol method of Galfré, G. and Milstein C.,
30 Methods Enzymol., 73: 3-46 (1981). Growth of hybridomas, selection in HAT medium, cloning and screening of hybridoma clones against specific antigens were carried out using standard methodology (Galfré, G. and Milstein C., Methods Enzymol., 73: 3-46 (1981), incorporated herein by
35 reference). Selected clones were injected into mice to produce large quantities of antibodies in

ascites (Galfré, G. and Milstein C., Methods Enzymol., 73: 3-46 (1981)) followed by purification of MAbs using protein A column chromatography (Bio-Rad, Hercules, CA).

5 Antibodies to Apo B-100

Conventional ways of producing MAbs to Apo B-100 include immunization of mice with LDL. This approach is convenient because it is relatively simple to isolate LDL. However, MAbs produced using LDL as an immunogen tend to be sensitive to conformational changes of Apo B-100 caused by variations in the lipid composition of LDL particles. For example, Apo B-100 epitopes are less reactive with a number of anti-Apo B MAbs due to the presence of various amounts of triglycerides (Keidar, S. et al., Metabolism, 39: 281-288 (1990); Galeano, N.F. et al., J. Biol. Chem., 269:511-519 (1994); Harduin, P. et al., Arterioscl. Thromb., 13: 529-535 (1993)).

20 For the methods and compositions described herein, an MAb is desired that fulfills two important criteria: (i) selective recognition of LDL and (ii) high and invariable reactivity with LDL particles, irrespective of possible variations in their lipid composition and/or conformation. Such an MAb must, therefore, recognize a stable, conformation-independent epitope which is uninfluenced by the lipid content and which is equally expressed in all LDL particles, but inaccessible in VLDL and chylomicrons. A MAb possessing these properties has not been previously described. For example, a detailed comparison of two known, potentially LDL specific MAbs demonstrated that neither of them can meet the above requirements (Milne, R. et al., J. Biol. Chem., 264:19754-19760 (1989); WO 93/18067). Cross-reactivity with VLDL, especially in samples

with high VLDL concentrations appears to be the major obstacle even in the case of most promising "anti-LDL" MAbs such as 8A2.1 and 4B5.6 (WO93/18067) (La Belle, M. et al., Clin. Chim. Acta, 191:153-160 (1990)). To obtain an anti-LDL MAb whose binding to LDL particles is not dependent on variations in LDL composition and/or conformation, mice were immunized with soluble Apo B-100 which had been delipidized, reduced, carboxymethylated and, purified by electrophoresis in polyacrylamide gels containing 8 M urea (Lee, D.M. et al., Biochim. Biophys. Acta, 666: 133-146 (1981)). Immunization with such delipidized, soluble, reduced, carboxymethylated, and electrophoretically purified Apo B-100 has not been previously reported.

The spleen cells of mice that were immunized using the soluble and electrophoretically purified Apo B, were then used to produce hybridomas according to standard hybridoma methods. A resulting MAb, HB₃CB₃, binds selectively to LDL particles produced by a hybridoma generated using spleen cells immunized with the soluble and electrophoretically purified Apo B.

HB₃CB₃ binds to the epitope near the T2 carboxy terminal region of B-100, exclusively, and does not recognize B-48. The epitope recognized by HB₃CB₃ may be conformationally changed or masked by lipids and/or other apolipoproteins present in VLDL.

Chylomicrons are not recognized by HB₃CB₃ because they lack Apo B-100. The HB₃CB₃ MAb, and LDL-binding fragments derived therefrom, can be used as an LDL-specific binding molecule in all of the compositions and methods described herein because of its specificity for LDL and lack of cross-reactivity with other lipoproteins.

Antibodies to Apo A-I

Two MABs raised against apolipoprotein A-I were selected from a library of MABs for developing rapid and sensitive means and methods of detecting lipoproteins predictive of risk of CHD. Both of them bind to HDL with a high affinity and show negligible reactivity with any other lipoprotein density class. The two anti-Apo A-I MABs, A1bD₅ and A1bE₂, bind to sterically distant epitopes since they do not compete with each other in their binding to either delipidized and purified Apo A-I or intact HDL particles. Both MABs to Apo A-I bind with high affinity to delipidized Apo A-I and to HDL and show negligible or no binding to LDL, VLDL, chylomicrons and Apos A-II, C-III and E as shown in Tables 1 and 2 below.

Antibodies to Apo A-II

An MAB to Apo A-II was produced using purified Apo A-II as an immunogen. This antibody binds with high affinity to HDL and is capable of removing all the HDL particles containing Apo A-II (LP-A-I:A-II particles) from plasma or serum, leaving the HDL particles without Apo A-II (LP-A-I particles) intact. This anti-Apo A-II MAB, CdB₅, is described by Koren, E. et al., Arteriosclerosis, 6:521a (1986); Alaupovic, P. et al., J. Lipid Res., 32:9-19 (1991).

Antibodies to Apo C-III

An MAB to Apo C-III, XbA₃, which is useful in quantification of VLDL particles is described by Koren, E. et al., Atherosclerosis, 95:157-170 (1992).

Antibodies to Apo E

Two MABs to Apo E are described by Koren, E. et al., Atherosclerosis, 95:157-170 (1992). One of them, EfB₁, binds preferably to Apo E associated with VLDL which are precipitated by heparin whereas

the other (E_fD₃) binds predominantly to Apo E in HDL which are not precipitated by heparin treatment of a sample.

II. MAbs Immobilized on Solid Phase Materials.

5 A. Dipsticks

Antibodies can be bound to a solid phase material for use in assays or purification procedures described herein. Various types of adsorptive materials, such as nitrocellulose, 10 Immobilon™, polyvinylidene difluoride (all from BioRad, Hercules, CA) can be used as a solid phase material to bind the anti-lipoprotein antibodies. Other solid phase materials, including resins and well-plates or other materials made of polystyrene, 15 polypropylene or other synthetic polymeric materials can also be used. In the preferred embodiment for assaying lipoprotein concentrations, pieces or strips of these materials are coated with one or more antibodies, or functional fragments 20 thereof, directed against specific epitopes of HDL, LDL, other lipoproteins, or apolipoproteins for use in patient samples. Such strips are referred to herein as "dipsticks". The dipsticks may also be attached to one end of a longer strip of a solid 25 support material, such as plastic, which can serve as a handle for dipping a dipstick into a solution or sample, such as a sample of whole blood, blood plasma, or blood serum. The plastic handle can also serve as a tether so that multiple dipsticks 30 can be attached to a common support. Such a multi-strip design may be particularly useful in a set-up for testing multiple lipoproteins and/or apolipoproteins simultaneously.

Although various sizes of dipsticks are 35 possible, typically, pieces of the solid phase material that are coated with antibody have the general dimensions of 0.5 cm x 0.5 cm and can be

attached to the longer solid support strips having general dimensions of 0.5 cm x 5 cm. Such dimensions permit an accurate determination of lipoprotein or apolipoprotein levels in as little as 100 μ l of blood.

The dipsticks described herein contain one or more regions containing immobilized antibodies specific for particular epitopes on apolipoproteins or lipoproteins.

10 **B. Coating solid phase material with antibodies**

Adsorption

 The strips of solid phase material, as used to make dipsticks, may be coated with antibodies by any of a variety of methods. If the strips are made of a protein-receptive solid phase material that adsorbs antibodies, such as nitrocellulose or polyvinylidene difluoride (PVDF) membrane, the material can be coated directly with antibody by immersing the solid phase material directly into a solution of antibody. However, a random interaction between the antibody molecules and the solid phase material can occur with this method and a certain percentage (up to 30 percent) of the antibody molecules that adsorb to the strips are immobilized in an orientation that makes their antigen-binding sites unavailable to bind their cognate lipoprotein or apolipoprotein antigen molecules.

30 Avidin-Biotin Complexes

 The proportion of antibody molecules on the dipsticks which are correctly oriented to bind their cognate antigens can be substantially increased if antibody molecules are attached to the solid phase material using avidin-biotin complexes. The strips of solid phase material are first coated with avidin or streptavidin (both available commercially, for example, from Sigma Chemical Co.,

5 avidin-coated strips are then incubated with antibody molecules which were previously biotinylated in their Fc domains (for example, using a biotin-LC-hydrazide labelling kit, Pierce, Rockford, IL). The avidin molecules adsorbed on
10 the solid phase material specifically bind the biotin linked to the Fc domains of the antibodies. In this way, the antibodies become attached to the solid phase material, optimally oriented with their carboxy terminal Fc regions linked to the surface
15 of the dipstick (via numerous biotin-avidin complexes) and with their antigen-binding domains directed away from the surface of the dipsticks and available for binding their cognate lipoprotein or apolipoprotein antigens in solution.

20 Alternatively, the same linkage can be achieved by chemically coupling biotin to the solid phase material and covalently attaching avidin to the Fc portion of the antibody molecules.

Use of the avidin (or streptavidin)-biotin system to coat strips with antibody yields dipsticks with a significantly higher capacity for binding lipoproteins and/or apolipoproteins than dipsticks which was made by simply applying antibody directly to the strips of solid phase material. The higher binding capacity of the dipsticks containing antibodies adsorbed to the solid phase using the avidin-biotin conjugation system results in a more sensitive dipstick. This is of a particular importance when dipsticks pre-stained with lipid or protein stains are used to capture lipoproteins, as described below.

After antibody has been adsorbed directly on the protein receptive dipstick material, or indirectly through avidin (or streptavidin), the strips are treated ("blocked") with a blocking agent in order to minimize nonspecific adsorption of lipoproteins, lipids, or apolipoproteins to unoccupied sites on the dipstick material. The dipsticks are treated with any of a variety of blocking agents such as bovine serum albumin (BSA), gelatin, Tris™, all of which are available commercially (Sigma, St Louis, MO) or nonfat milk proteins. For example, avidin-coated PVDF strips can be blocked with 2 percent (w/v) milk blocking solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 48 hours at 4°C.

Antibodies can also be chemically coupled to the substrate to form the dipsticks.

C. Design of dipsticks

A dipstick may contain more than one antibody so that the single dipstick can be used to detect more than one apolipoprotein or lipoprotein. For example, two or more separate pieces of a solid phase material, each coated with an antibody directed against a particular apolipoprotein or lipoprotein, can be attached to a longer strip of solid support to produce a dipstick with two or more separate areas, each specific for a particular lipoprotein or apolipoprotein. The means to attach the solid phase material to a solid support should not impair the function of the molecules coated on the solid phase material and must be secure enough to withstand soaking in whole blood, serum, plasma, and the other solutions described herein which are used to wash, stain, and preserve the dipsticks. A preferred method of attaching antibody-coated solid phase material to a longer strip of solid support is to use a glue or cement such as an acrylate

adhesive (for example, SUPER GLUE™, Super Glue Corporation, Hollis, NY; DURO™, Loctite Corporation, Cleveland, OH).

Dipsticks can be designed for quantification of one or more lipoproteins or apolipoproteins in a blood sample. In one embodiment, dipsticks designed for quantification of a lipoprotein or apolipoprotein contain a single antigen-binding area which is dipped into a blood sample, stained for bound lipid lipoproteins or apolipoprotein, and visually compared with a set of printed colored standards to determine the concentration of the particular lipoprotein or apolipoprotein.

In addition, dipsticks can be designed for detecting a change in the relative level of particular lipoproteins or apolipoproteins in a blood sample. Dipsticks can be designed for detecting a change in the relative level of specific lipoproteins or apolipoproteins which contain two antigen-binding areas, each area coated with a different antibody. After processing the dipstick to detect the lipoprotein or apolipoprotein antigens bound by each antibody, the relative intensities of the colors in the two areas of the dipstick are compared as an indication of the relative concentrations of the two antigens in the blood.

A determination of relative levels of specific lipoproteins or apolipoproteins can also be made by simultaneously using two separate dipsticks. However, a single dipstick with two antigen binding areas is generally easier to use, especially for the lay person, and an assessment of relative color intensities in two areas in close proximity on a single dipstick is relatively easy to make even for the untrained observer.

A simple comparison of relative color intensities in two areas may be sufficient for an assessment of an increase or decrease in a lipoprotein or apolipoprotein ratio. However, in a preferred embodiment, each doublet of stained areas is also compared with printed colored standards covering an appropriate range of ratios of color intensities.

In another embodiment, dipsticks are made that contain distinct areas or spots of known amounts of molecules whose levels are to be determined by the dipstick. For example, known amounts of lipid, lipoproteins and/or apolipoproteins are placed on the dipsticks using methods such as those used for attaching antibodies to the solid phase material described above. Such known amounts of lipids, lipoproteins, and apolipoproteins present on dipsticks act as "internal standards", whose staining intensity can be compared to that in the antigen-binding areas of the dipstick in order to estimate the amount of antigen bound by the antibodies on the dipstick.

D. Storage of dipsticks

Although there is a possibility that some antibodies could be adsorbed onto a solid phase material, dried, and subsequently rehydrated without significant loss of their binding capacity, most of the antibodies on dipsticks are better preserved if stored in at least a small amount of aqueous buffer, such as phosphate buffered saline (pH 7.4), in order to retain their binding capabilities. For example, the dipsticks can be stored damp in sealed foil or plastic bags containing enough buffer to prevent dehydration. The buffer may also contain an appropriate quantity (25 to 50 percent) of a stabilizing agent such as glycerol or sucrose. The dipsticks in the sealed

bags can be stored in such buffers at temperatures ranging generally from 4 to 25°C, for up to three months without significant loss of accuracy. The dipsticks should be removed from the storage bag immediately prior to use, and rinsed for 30 seconds under tap water or physiological buffer (for example, PBS) (at a temperature less than 40°C to avoid denaturation of the immobilized MAb) in order to remove residual stabilizing agents and storage buffer.

III. Determination of Lipoprotein Concentrations using Solid Phase Assays.

The crucial reagents in this approach are the antibodies or functional fragments of the antibodies, which specifically recognize and bind a particular lipoprotein, leaving other lipoproteins in the sample unadsorbed. Many assays are known in which the antibodies are immobilized onto a solid support, ranging from the walls of test tubes or well plates to polymeric beads, such as agarose, acrylamide or ion exchange resins.

In order to assay a sample of whole blood, serum or plasma for HDL or LDL using dipsticks, dipsticks are incubated with EDTA-treated or heparinized blood for 2 to 5 minutes at room temperature. After incubation, each strip is washed to remove unbound blood, (for example, under tap water for 0.5 to 1 minute at temperatures not exceeding 40°C. The dipsticks are then stained, for example, by immersing the dipsticks in a solution of stain such as Sudan Red 7B for 2 to 5 minutes at room temperature to stain the lipid present in the bound lipoprotein particles. Excess stain is then removed by an additional wash. Residual moisture or stain may be drawn off by touching an absorbent towel with the edge of dipstick. The "face" of the dipstick, that is, the side of the dipstick containing immobilized

antibody, should not be blotted, which might disturb the immobilized antibody and/or bound antigen. After drying, the intensity of the staining can be compared with standardized colored strips to determine the concentration of lipoprotein in the blood.

A number of other lipid stains such as Oil Red O or Sudan Black B can be also used for staining of dipsticks. However, in the preferred embodiment, Sudan Red 7B, also known as Fat Red 7B (Sigma, St. Louis, MO), dissolved in a mixture of methanol and NaOH is used because of its high color intensity. In another embodiment lipoproteins are stained prior to being bound to antibody ("pre-stained"), such as antibody on a dipstick, using any of the above mentioned lipid stains dissolved in propylene glycol (Wollenweber, J. and Kahlke, W., Clin. Chim. Acta, 29:411-420 (1970)). The pre-stained blood, plasma or serum sample is then incubated, for example, with anti-LDL or anti-HDL dipsticks. After washing and drying, the quantity of pre-stained lipoprotein captured by the dipstick is determined visually according to the intensity of the color, for example, by comparison with a set of printed colored standards.

IV. Determination of Apolipoprotein Concentrations Sandwich Assays

The methods described above for the detection of lipoproteins depend on the staining of lipids associated with the lipoproteins which have been bound by a lipoprotein-specific antibody on the dipstick. The determination of the concentration of a specific apolipoprotein in blood samples requires a "sandwich" method of detection in which at least two anti-apolipoprotein antibodies with distinct specificities for two different epitopes of the same apolipoprotein are used. In a

preferred embodiment, two MABs are used that bind to separate epitopes of the apolipoprotein. One of the two MABs is conjugated to an enzyme, for example, horseradish peroxidase, alkaline

5 phosphatase, or to biotin which in turn binds to an avidin- or streptavidin-enzyme conjugate of an enzyme-based chromogenic labeling system. The enzyme-conjugated anti-apolipoprotein MAB is added to and mixed with the blood, serum or plasma

10 sample. The second MAB is immobilized on a dipstick. During the incubation with the blood sample, typically 10 minutes at room temperature), the enzyme-conjugated MAB binds to its cognate epitope and forms soluble antibody-antigen complex.

15 The dipstick is then immersed into the blood sample and incubated for 2 to 5 minutes at room temperature to allow the immobilized MAB to bind the other epitope of the same apolipoprotein. The dipstick is then removed and washed as described

20 above. After washing, the strip is immersed into a solution containing the appropriate chromogenic substrate (2 to 5 minutes at room temperature) for the enzyme that was conjugated to the first MAB for example, 3,3',5,5'-tetramethylbenzidine ("TMB") or

25 4-chloro-1-naphthol for horseradish peroxidase; or 5-bromo-4-chloro-3-indolyl phosphate for alkaline phosphatase. The dipstick is washed, dried, and the color developed and compared with color standards which correspond to various concentrations of

30 apolipoprotein in the blood sample.

Alternatively, the dipstick can first be incubated with a blood sample for 2 to 5 minutes to bind the apolipoprotein and then washed and immersed into the solution of the MAB-enzyme

35 complex for 10 minutes at room temperature. After an additional washing, the dipstick is immersed

into a solution of chromogenic substrate and stained as explained above.

For example, in a noncompetitive "sandwich" version of ELISA, anti-LDL MAb is adsorbed to the wells of a microtiter plate. A plasma or serum sample is added to wells of a microtiter plate coated with anti-LDL MABs. The sample is incubated in the wells to allow the anti-LDL MAb to bind LDL in the plasma or serum sample. The unbound components of the sample are then removed and the quantity of LDL-Apo B captured by the HB₃CB₃ MAb is determined using a Pan B (D₆) MAb-peroxidase complex and chromogenic peroxidase substrate as described above. Peroxidase labeled polyclonal antibody to Apo B may be used instead of Pan B MAb.

In a competitive variation of ELISA, anti-LDL HB₃CB₃ MAb is mixed with a sample of plasma or serum and allowed to bind to LDL. This mixture is then added to the ELISA microtiter plate coated with LDL. MAB molecules that did not react with LDL in the sample are free to bind to the layer of LDL immobilized on the plate. The higher the concentration of LDL is in the plasma or serum sample, the less anti-LDL MAb will bind to LDL on the plate. The quantity of anti-LDL MAb bound to the plate is determined using any of the commercially available enzyme-conjugated secondary antibodies such as, alkaline phosphatase or peroxidase conjugated to goat anti-mouse IgG, (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and subsequent incubation with an appropriate chromogenic substrate. Alternatively, the anti-LDL MAb can be conjugated to an enzyme, for example to form an anti-LDL MAb-peroxidase or alkaline phosphatase complex, and thereby eliminate the use of an enzyme-conjugated secondary antibody, with the same results.

In an alternate embodiment of the sandwich method, only one of the antibodies to the particular apolipoprotein is an MAb and the other antibody is a polyclonal anti-apolipoprotein antibody. This method can work as well as the two MAb sandwich method described above, if the one MAb is specific for the particular apolipoprotein of interest, that is, does not cross-react with other apolipoproteins. Either the MAb or the polyclonal antibody may be the immobilized or the enzyme-conjugated antibody in this embodiment of the sandwich method. This method is most accurate when the MAb (whether enzyme-conjugated or immobilized) is allowed to bind to the apolipoprotein antigen first, and the polyclonal anti-apolipoprotein is allowed to bind to the apolipoprotein second. This stepwise procedure prevents underestimation of the quantity of the particular apolipoprotein in the blood sample by insuring 1) that none of the polyclonal antibody molecules are given the first opportunity to bind, and thereby block, the specific epitope recognized by the MAb molecules and 2) that essentially only those apolipoprotein molecules recognized by the MAb are detected. The highly specific anti-Apo B MAb HB₃CB₃ described herein is thus an example of an MAb useful in any of the above-described sandwich methods as applied to the detection and quantification of Apo B.

The above described sandwich method, to determine the amount of apolipoprotein in a sample, is useful not only for quantification of single apolipoproteins, but also for analysis of ratios between various apolipoproteins and lipoproteins in which case dipsticks with two or more antigen binding areas are used as described above.

In another embodiment, any of the above described enzyme-conjugated monoclonal or

polyclonal anti-apolipoprotein antibodies is replaced with a "stained" antibody, that is, an antibody coupled with a protein stain such as nitro blue tetrazolium (Glenner, G.G. Formazans and Tetrazolium Salts, In: H.J. Conn's Biological Stains, pp. 225-235. The Williams and Wilkins Company USA 1990; US. Patent 4,786,589)). Such a "stained" or "colored" antibody is mixed with a blood, plasma or serum sample to bind, and thereby, pre-stain the antibody's cognate apolipoprotein or lipoprotein in the sample. An antibody coated dipstick is then immersed into the sample in order to absorb the lipoprotein which has been pre-stained via binding to the stained antibody. After washing and drying the quantity of pre-stained lipoprotein is determined visually by comparing the color of the dipstick with a set of printed color standards.

V. Use of Antibody in Solution

Immunoprecipitation is another means of identifying small amounts of protein in a complex mixture by its interaction with antibody. The amount of antigen present can be determined by changes in turbidity of a solution using optical detection means such as a spectrophotometer, or the precipitate isolated and measured by detection of label on the antibody, typically using ELISA, measurement of a fluorescent label or measurement of a radiolabel. In those cases where the antibody does not precipitate antigen, precipitation may be enhanced through the use of a second anti-antibody or a second antibody immunoreactive with the same antigen.

For example, in an immunoturbidimetric assay for LDL, one preferably would use a single monoclonal antibody capable of precipitating exclusively LDL. Single MABs generally do not

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precipitate the antigens they are immunoreactive with. Accordingly, two or more Mabs immunoreactive with the same antigen can be used to precipitate the antigen. For quantitation of LDL, one would

5 use two MABs which are specific for LDL. Useful antibodies include HB3cB3 combined with another antibody such as WbA53aC1-A6, described by Koren, et al. Biochemistry 26, 2734-2740 (1987). This results in immunoprecipitation of LDL without

10 affecting other plasma lipoproteins such as VLDL and HDL.

In addition, the above-described sandwich method can be used to detect any blood protein of interest in a particular sample, provided, as

15 described above, that either two distinct MABs are available which do not interfere with each other's binding to the particular protein, or one MAb and a polyclonal antibody are available for the particular protein and the MAb is allowed to bind

20 to the particular protein before the polyclonal antibody.

As noted above, anti-LDL MABs, such as HB₃cB₃, are useful for quantification of LDL-cholesterol in antibody-antigen precipitation techniques and

25 enzyme-linked immunosorbent assays (ELISA). For example, in a precipitation method the anti-LDL MAB is added to human serum or plasma and allowed to bind to LDL. The immune complex of LDL bound to anti-LDL MAB is then precipitated by mixing in an

30 excess amount of protein A or an anti-mouse IgG polyclonal antibody. Precipitation of the complexes is enhanced by centrifuging the mixture and then discarding the supernatant. The precipitate containing LDL is then washed and dissolved in

35 8 M urea in PBS or treated with detergents such as Triton X-100 and cholic acid (Sigma, St. Louis, Mo). This is followed by determination of LDL-

cholesterol using an enzymatic assay for cholesterol (Sigma, St. Louis, MO).

5 MABs or polyclonal antibodies specific for LDL can be labeled with any of a number of fluorescent compounds such as fluorescein isothiocyanate, europium, lucifer yellow, rhodamine B isothiocyanate (Wood, P. In: Principles and Practice of Immunoassay, Stockton Press, New York, pages 365-392 (1991), incorporated herein by
10 reference) for use in immunoassays. In conjunction with the known techniques for separation of antibody-antigen complexes, these fluorophores can be used to quantify LDL. The same applies to chemiluminescent immunoassay in which case either
15 anti-LDL MAB or LDL can be labeled with isoluminol or acridinium esters (Krodel, E. et al., In: Bioluminescence and Chemiluminescence: Current Status. John Wiley and Sons Inc. New York, pp 107-110 (1991); Weeks, I. et al., Clin. Chem. 29:1480-1483 (1983), incorporated herein by reference).
20 Radioimmunoassay (Kashyap, M.L. et al., J. Clin. Invest. 60:171-180 (1977)) is another technique in which anti-LDL MAB can be used after coupling of anti-LDL MAB or LDL with a radioactive isotope such
25 as ^{125}I . Some of these immunoassays can be easily automated by the use of appropriate instruments such as the IMXTM (Abbott, Irving, TX) for a fluorescent immunoassay and Ciba Corning ACS 180TM (Ciba Corning, Medfield, MA) for a chemiluminescent
30 immunoassay.

VI. Purification of Apolipoprotein

Although described herein with reference to the use of the specific anti-apolipoprotein antibodies for diagnostic purposes, the antibodies
35 can be immobilized to resins or well plates or other inert substrates for use in purification of the apolipoprotein which the antibody is

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immunoreactive with. Antibodies specific for LDL can also be used to make immunoaffinity columns in which anti-LDL MAb is conjugated to a solid support. In addition to use in LDL purification, such immunoaffinity columns can be used to selectively remove LDL from a patient's blood using an extracorporeal circulation device. In a preferred embodiment, the antibody is bound to an acrylamide or agarose resin particulate such as SEPHAROSE™ (Pharmacia Fine Chemicals, Piscataway, NJ) or Affi-GEL™, (Bio-Rad, Hercules, CA), and placed in a chromatography column. The sample from which the apolipoprotein is to be purified is applied to the column, the material which is not bound by the antibody is washed from the column, and the bound lipoprotein is eluted from the antibody using a salt gradient or other standard technique.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Determination of Binding Specificity of Apolipoprotein MAbs.

To determine the binding specificity of the anti-Apo A-I MAb AIbD₅, ELISA plates were coated with antigens using concentrations indicated in the left column of Table 1, below. Each antigen was incubated with the AIbD₅ MAb (10 µg/ml) followed by washing and detection of the bound MAb with a goat anti-mouse-peroxidase conjugate. Each number represents an average optical density from three separate experiments. AIbD₅ MAb bound strongly to Apo A-I and HDL, but exhibited no significant binding to other antigens.

Table 1: Binding Specificity of A1bD5 Mab (anti-Apo A-I) to apolipoprotein and lipoproteins.

Antigen (μ g protein per ml)	Apolipoproteins			Chylo- microns	Lipoproteins		
	A-I	A-II	C-III		VLDL	LDL	HDL
80	1.620	0.105	0.084	0.090	0.153	0.205	0.211
40	1.012	0.090	0.060	0.071	0.095	0.132	0.130
20	0.841	0.080	0.053	0.060	0.080	0.095	0.098
10	0.520	0.075	0.032	0.045	0.080	0.090	0.087
5	0.210	0.063	0.040	0.038	0.075	0.080	0.075
2.5	0.135	0.020	0.020	0.018	0.060	0.047	0.050
							0.268

The epitopes recognized by the MABs A1bD₅ and A1bE₂ are different and sterically separated since these antibodies did not compete with each other when allowed to bind simultaneously to HDL.

The epitopes recognized by the MABs A1bD₅ and A1bE₂ are different and sterically separated since these antibodies did not compete with each other
15 when allowed to bind simultaneously to HDL.

Table 2: Binding to AbE, Mab (anti-Apo A-I) to apolipoproteins and lipoproteins

Antigen (μ g protein per ml)	Apolipoproteins				Lipoproteins			
	A-I	A-II	C-III	E	Chylomicrons	VLDL	LDL	HDL
80	1.205	0.095	0.061	0.076	0.107	0.183	0.200	1.431
40	0.780	0.080	0.060	0.053	0.103	0.115	0.163	1.108
20	0.337	0.083	0.065	0.060	0.098	0.108	0.108	0.860
10	0.340	0.071	0.047	0.059	0.080	0.083	0.099	0.495
5	0.189	0.070	0.053	0.045	0.063	0.070	0.063	0.231
2.5	0.105	0.068	0.048	0.040	0.060	0.058	0.060	0.150

**Example 2: Production of Anti-Apo B-100
 Antibody, HB₃CB₃.**

The MAb to Apo B, HB₃CB₃, was produced by immunizing mice with Apo B-100 molecules which had been delipidized, reduced, carboxymethylated, and purified by electrophoresis on a polyacrylamide gel containing 8 M urea. Delipidized Apo B-100 readily precipitates due to self-aggregation in aqueous media. In addition to the self-aggregation, Apo B-100 is also susceptible to fragmentation during the solubilization procedure (Socorro, L. and Camejo, G.J. Lipid Res., 20:631-645 (1979); Olofsson, S.O. et al., Biochemistry, 19:1059-1064 (1980)). Therefore, in order to separate self-aggregated and degraded material from the preserved protein, the delipidized, reduced, and carboxymethylated Apo B-100 was electrophoresed on a polyacrylamide gel containing 8 M urea. Coomassie blue staining of the urea-polyacrylamide gel revealed three distinct bands. The most prominently stained band in the urea-containing polyacrylamide gel was cut out immediately after the completion of electrophoresis and subcutaneously injected (while still in the gel) into mice without further manipulation of addition or adjuvants. The most prominently stained band on the urea-polyacrylamide gel had previously been shown to be pure Apo B-100, as confirmed by eluting the band from the urea-containing gel and electrophoresing it under reducing and denaturing conditions on a standard SDS-containing polyacrylamide gel. The SDS-gel revealed a single protein band of the expected mobility of Apo B-100.

Approximately 10 to 20 µg of the Apo B-100 band excised from the urea-containing gel was injected four times at various locations over a period of two months. The mice immunized with the Apo B-100 according to this procedure were then

used in standard methods to produce hybridomas. Out of forty-two hybridomas which produced MABs that bound Apo-B-100, only one, HB₃CB₃, produced a MAB that bound exclusively to LDL, as shown in

5 Table 3, below.

To characterize the binding specificity of the HB₃CB₃ MAB, ELISA plates were coated with lipoproteins using concentrations indicated in the left column of Table 3 below. Each antigen was
10 incubated with the HB₃CB₃ MAB (10 µg/ml) followed by washing and detection of the bound MAB with a goat anti-mouse IgG-peroxidase conjugate. Each number represents an average optical density reading from three separate experiments. HB₃CB₃ MAB showed a
15 strong and exclusive binding to LDL. Identical results were obtained with competitive ELISA (see below) in which the binding of HB₃CB₃ MAB to LDL absorbed to the wells of an ELISA plate was found to be inhibited only by LDL.

Table 3: Binding Specificity of HB₃CB₃ MAB (anti-Apo B-100) to Lipoproteins

Antigen (µg protein per ml)	Lipoproteins			
	Chylo- microns	VLDL	LDL	HDL
80	0.085	0.098	1.900	0.078
40	0.081	0.095	1.432	0.080
20	0.072	0.084	1.003	0.082
10	0.060	0.068	0.605	0.075
5	0.043	0.063	0.211	0.060
2.5	0.040	0.051	0.140	0.060

HB₃CB₃ MAB binds to Apo B-100 in Western blots and shows no significant reactivity with any other plasma apolipoproteins or proteins. Western
5 blotting also reveals that HB₃CB₃ MAB binds to the so-called T₂ fragment of Apo B-100 which represents a carboxy terminal 1,287 amino acid piece of Apo B-

100 (Cardin, A.D. et al. J. Biol. Chem., 259: 8522-8528 (1984)). The HB₃CB₃ MAb recognizes an epitope outside of the receptor binding domain localized at the amino terminus of the T₂ fragment because it
5 does not interfere with the binding of LDL to the LDL receptor on cultured human skin fibroblasts and human hepatoma HepG2 cells.

HB₃CB₃ MAb binds strongly and specifically to LDL with little or no significant reactivity with
10 VLDL (Table 3, above). Furthermore, immunoaffinity chromatography of human serum using HB₃CB₃ MAb immobilized on an AFFI-GEL™ column (Bio-Rad, Hercules, CA) always yields a lipoprotein fraction with typical β electrophoretic mobility, free of
15 any other lipoproteins. Identical results were obtained with normal, hypertriglyceridemic as well as hypercholesterolemic sera as determined using a commercial lipoprotein electrophoresis kit (Ciba Corning, Medfield, MA). In addition, crossed
20 immunoelectrophoresis (Koren, E., et al., Biochemistry, 21:5347-5351 (1982)), of the lipoproteins retained by the HB₃CB₃ column revealed only one symmetrical Apo B peak very similar in shape and mobility to ultracentrifugally isolated
25 LDL. These immunoaffinity chromatography results confirmed the specificity of HB₃CB₃ MAb for LDL as well as the lack of reactivity with VLDL.

Further evidence for the LDL specificity of HB₃CB₃ MAb came from a comparison between the LDL-
30 Apo B concentrations as determined in human sera using an ELISA with HB₃CB₃ MAb and the concentrations of LDL-cholesterol determined using a commercially available LDL-cholesterol assay kit (Sigma, St. Louis, MO). In the competitive ELISA
35 method, the wells of microtiter plates were coated with LDL and blocked with 0.1% nonfat milk proteins (Kirkegaard Perry Laboratories, Gaithersburg, MD).

This was followed by incubating dilutions of human sera with HB₃CB₃ MAb for 18 hours at 4°C. These mixtures were then pipetted into wells coated with LDL and incubated for 3 hours at room temperature.

5 During this time, HB₃CB₃ MAb molecules that did not previously bind to LDL in the serum, bound to the LDL coating the plate. The quantity of HB₃CB₃ MAb that was bound to LDL on the plate was inversely proportional to the concentration of LDL in the

10 serum sample. After washing off the unbound components, bound HB₃CB₃ was detected using peroxidase-labeled anti-mouse IgG and the chromogenic (ABTS) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Intensity of

15 developed color was determined using an ELISA plate reader MR 580 (Dynatech, Chantilly, VA). Dilutions of pure LDL (isolated by ultracentrifugation, see Alaupovic, P. et al., Biochim. Biophys. Acta, 260: 689-707 (1972), incorporated herein by reference)

20 with known concentrations of ApoB-100 were used on each plate to construct a standard curve from which the concentrations of LDL-Apo B in the serum samples were calculated. The LDL-cholesterol concentrations were determined in the same sera

25 using the LDL-DIRECT™ commercial kit (Sigma, St. Louis, MO). This method was used because it allows for an accurate determination of LDL-C even in sera with triglycerides as high as 1139 mg/dl.

Based on analysis of 100 human sera with

30 variable lipoprotein profiles, the correlation between LDL-Apo B values determined by the HB₃CB₃ ELISA and LDL-cholesterol values determined by the commercial kit was highly significant. The correlation coefficient was 0.94 and corresponding

35 P value was <0.0001. Twenty-six of these sera contained very high levels of triglycerides (400 to 1125 mg/dl), and were therefore rich in VLDL.

However, the presence of excess VLDL did not interfere with the selective recognition of LDL by the HB₃CB₃ MAb. The correlation between LDL-Apo B determined by the HB₃CB₃ ELISA and the LDL-C determined by the commercial kit (Sigma, St. Louis, MO) was highly significant ($r=0.93$, $p<0.0001$) even in this subgroup of twenty-six hypertriglyceridemic sera.

The above data clearly indicated that HB₃CB₃ MAb recognizes an epitope of Apo B-100 that is fully expressed only on LDL particles. The HB₃CB₃ hybridoma cells producing the antibody were deposited in the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852) under the ATCC designation number HB11612.

Example 3: Preparation of Anti-LDL and Anti-HDL Dipsticks to Assay Human Serum, Plasma and Whole Blood.

To prepare dipsticks for analyses of LDL and HDL in human whole blood, serum and plasma samples, PVDF membrane (Bio-Rad, Hercules, CA) was treated with methanol and washed with water according to manufacturer's instructions. Washed membrane was cut into strips (5 x 60 mm) and stored in phosphate buffered saline (Sigma, St. Louis, MO) pH 7.4 at 4°C. The strips were incubated with the anti-LDL MAb HB₃CB₃ or the anti-HDL MAb AIbD₅ in PBS. Both of these MAbs were adjusted to the concentration of 1 mg/ml. Each strip was incubated in 6 ml of an antibody solution for 24 hours at 4°C followed by two additional 24-hour incubations using fresh antibody solutions each time to adsorb MAbs to each strip. The purpose of these sequential incubations was to saturate strips with adsorbed antibodies. This was accomplished by the three consecutive incubations as indicated by the concentrations of antibodies left in solution after each incubation with PVDF strips. Coating of strips with MAbs was

followed by an incubation in a 2% solution of nonfat milk proteins in PBS (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 24 hours at 4°C to block areas of PVDF not occupied by antibody molecules. After three washes in 30 ml of PBS, the strips were kept in PBS at 4°C up to two weeks without noticeable loss of activity. Antibody-coated and blocked strips were also immersed in PBS containing 50 percent sucrose (Sigma, St. Louis, MO) for 5 minutes and sealed in small plastic bags. The sealed dipsticks retained their capacity to bind lipoproteins for up to twelve weeks at 4°C.

Example 4: Use of Dipsticks to Assay LDL and HDL by Lipid Staining and Comparison to Other Assay Method.

Samples of human blood serum or plasma were diluted with 0.5% EDTA solution by adding 100 μ l of EDTA to 100 μ l of sample in an 0.5 ml plastic tube. A small piece of antibody-coated dipstick (5 x 5 mm) was immersed into diluted serum for 2 minutes at room temperature. The tube was shaken occasionally two to three times. This was followed by washing off unbound constituents of serum with tap water for 1 minute. The strip was then gently blotted against paper tissue to remove excess water and air dried for 2 minutes. Washing and drying was followed by staining of the strip in 200 μ l of 0.02% Sudan Red 7B (Sigma, St. Louis, MO) dissolved in a mixture of methanol and 0.1 M NaOH (5:1 volume: volume) for 3 minutes with occasional shaking. The staining was followed by washing under the tap water for 1 minute, blotting and air drying for 5 minutes. A dipstick coated with no antibody and blocked with nonfat milk proteins served as a negative control. The whole procedure lasted approximately 15 minutes and was carried out at room temperature. Sudan Red 7B stained the lipid

moiety of lipoproteins (LDL and HDL, respectively) captured on the antibody-coated strips. The intensity of color was clearly proportional to concentrations of LDL and HDL cholesterol determined in each serum by the respective conventional methods (Sigma, St. Louis, MO), as shown in Tables 4 and 5 below.

In Table 4, serum LDL-cholesterol was determined in all samples by a direct LDL-C assay (Sigma, St. Louis, MO). The same serum samples also were incubated with anti-LDL dipsticks and stained with Sudan Red 7B as described above. The color intensity was assessed visually (on an arbitrary scale of 1 to 15) by three individuals (I, II and III) presented with the complete set of 16 dipsticks at the same time. The averaged score of the color intensity correlated significantly with LDL-C concentrations ($r=0.97$, $p<0.0001$).

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sample number	Serum LDL-cholesterol (mg/dl)	Dipstick color intensity			Average color score
		I	II	III	
1	100	1	1	1	1
2	100	1	1	1	1
3	100	1	1	1	1
4	100	1	1	1	1
5	100	1	1	1	1
6	100	1	1	1	1
7	100	1	1	1	1
8	100	1	1	1	1
9	100	1	1	1	1
10	100	1	1	1	1
11	100	1	1	1	1
12	100	1	1	1	1
13	100	1	1	1	1
14	100	1	1	1	1
15	100	1	1	1	1
16	100	1	1	1	1
17	100	1	1	1	1
18	100	1	1	1	1
19	100	1	1	1	1
20	100	1	1	1	1
21	100	1	1	1	1
22	100	1	1	1	1
23	100	1	1	1	1
24	100	1	1	1	1
25	100	1	1	1	1
26	100	1	1	1	1
27	100	1	1	1	1
28	100	1	1	1	1
29	100	1	1	1	1
30	100	1	1	1	1
31	100	1	1	1	1
32	100	1	1	1	1
33	100	1	1	1	1
34	100	1	1	1	1
35	100	1	1	1	1
36	100	1	1	1	1
37	100	1	1	1	1
38	100	1	1	1	1
39	100	1	1	1	1
40	100	1	1	1	1
41	100	1	1	1	1
42	100	1	1	1	1
43	100	1	1	1	1
44	100	1	1	1	1
45	100	1	1	1	1
46	100	1	1	1	1
47	100	1	1	1	1
48	100	1	1	1	1
49	100	1	1	1	1
50	100	1	1	1	1
51	100	1	1	1	1
52	100	1	1	1	1
53	100	1	1	1	1
54	100	1	1	1	1
55	100	1	1	1	1
56	100	1	1	1	1
57	100	1	1	1	1
58	100	1	1	1	1
59	100	1	1	1	1
60	100	1	1	1	1
61	100	1	1	1	1
62	100	1	1	1	1
63	100	1	1	1	1
64	100	1	1	1	1
65	100	1	1	1	1
66	100	1	1	1	1
67	100	1	1	1	1
68	100	1	1	1	1
69	100	1	1	1	1
70	100	1	1	1	1
71	100	1	1	1	1
72	100	1	1	1	1
73	100	1	1	1	1
74	100	1	1	1	1
75	100	1	1	1	1
76	100	1	1	1	1
77	100	1	1	1	

1	145	4	4	4	4.0
2	130	4	4	3	3.7
3	97	2	2	2	2.0
4	165	6	5	4	5.0
5	115	3	2	2	2.3
6	200	8	10	9	9.0
7	207	8	8	9	8.3
8	160	6	5	5	5.3
9	115	2	3	2	3.3
10	276	15	15	15	15.0
11	155	6	5	5	5.3
12	98	2	1	1	1.3
13	123	3	3	3	3.0
14	130	3	4	2	3.0
15	185	6	6	7	6.3
16	73	1	1	1	1.0

In Table 5, serum HDL-cholesterol was determined in all samples by Sigma's HDL-C kit. The same serum samples were incubated with anti-HDL dipsticks and stained with Sudan Red 7B as described above. The color intensity was assessed visually (on an arbitrary scale of 1 to 10) by three individuals (I, II and III) presented with the complete set of 9 dipsticks at the same time. The averaged score of the color intensity correlated significantly with HDL-C concentrations ($r=0.93$, $p<0.0005$)

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Table 5: Correlation between the serum HDL-cholesterol concentration and the color intensity of AIBD₅ coated (anti-HDL) dipsticks stained with Sudan Red 7B.

Sample number	Serum LDL-cholesterol (mg/dl)	I	II	Dipstick color intensity III	Average color score
1	65	10	10	10	10.0
2	53	6	6	6	6.0
3	58	5	6	6	5.7
4	48	5	5	6	5.3
5	40	5	6	5	5.3
6	60	7	8	8	7.7
7	28	1	1	1	1.0
8	37	3	3	3	3.0
9	39	3	3	3	3.0

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Two other lipid stains, Oil Red O and Sudan Black B, gave similar results. All of these stains are commonly used for staining of lipoproteins in electrophoretic analyses of serum (Stein E.A. and Meyers, G.L., Lipids, Lipoproteins and Apolipoproteins, In Tietz Textbook of Clinical Chemistry, W.B. Saunders, Philadelphia pp 1002-1093 (1994)). The color on dipsticks is stable for fourteen days. Anti-LDL dipsticks were more intensely colored than anti-HDL dipsticks which reflects higher lipid content per LDL particle. The above experiments were also carried out with human plasma and serum with identical results. The total time to run the dipstick assay, from insertion into a blood sample to development of color is approximately 15 minutes.

Example 5: Stability of Dipsticks and Lipid Stains.

Anti-LDL and anti-HDL dipsticks were stored in 50 percent sucrose in sealed plastic bags for 3, 6 and 12 weeks at 4°C as described above. Dipsticks were washed under the tap water for 1 minute and used immediately after removal from plastic bags. Incubations with serum and staining were carried out as described above at each of the indicated time intervals. To avoid storage-related decline in serum lipoproteins concentrations, aliquots of serum were stored at -70°C and thawed at indicated time intervals immediately prior to experiments with dipsticks. There were no noticeable differences between anti-LDL dipsticks stored for various times over a period of twelve weeks. Similar results were obtained with anti-HDL dipsticks. All three lipid stains (Sudan Red 7B, Sudan Black B and Oil Red O) dissolved in methanol were stable for four months at room temperature as well as 4°C. However, the 0.1 M NaOH solution, which is present in each staining solution, must be

added immediately prior to staining the dipsticks to assure optimal and reproducible results.

Example 6: Use of the Avidin-Biotin Complex to Bind Antibody to Dipsticks.

5 The avidin-biotin system was also used to bind antibody molecules to PVDF strips. PVDF strips were incubated with egg-yolk avidin (Sigma, St. Louis, MO) dissolved in PBS (3 mg/ml) for 24 hours at 4°C. This step was repeated for a total of
10 three times using fresh avidin solution each time. Strips were then blocked with 2% nonfat milk for 24 hours at 4°C. The strips were then incubated three times for 24 hours at 4°C in a solution of biotinylated anti-LDL HB₃CB₃ MAb (1 mg/ml). The
15 anti-LDL MAb was biotinylated at its Fc fragment using the periodate-biotin- LC-hydrazide technique (Pierce, Rockford, IL) which covalently couples biotin molecules to carbohydrate residues concentrated at the Fc portion of the antibody
20 molecule. Biotinylation carried out in this fashion leaves the antigen combining sites of an antibody intact. Furthermore, with the Fc portion of the antibody attached to the layer of avidin on the strip, the antigen-binding sites are free to
25 bind the antigen.

The avidin-biotin anti-LDL strips made with the avidin-biotin complex were kept in PBS at 4°C. These anti-LDL dipsticks made with avidin-biotin complexes stained more intensely than dipsticks
30 coated with non-biotinylated anti-LDL. Each avidin molecule consisting of four subunits can bind four biotin molecules and the affinity of binding between these two molecules is extremely high (Savage, M.D. et al. Avidin-Biotin Chemistry: A
35 Handbook, Pierce Chemical Company, Rockford, IL (1992)). Thus, anti-LDL dipsticks made with avidin-biotin complexes exhibited a higher LDL binding capacity than dipsticks made without the

avidin-biotin system to bind antibody to the dipsticks.

Example 7: Dipstick Method to Assay LDL by staining of Apolipoprotein B.

5 The Pan B D₆ MAb is specific for Apo B and binds equally well to all Apo B-containing lipoproteins including LDL. The binding of Pan B (D₆) MAb to LDL does not interfere with the binding of HB₃CB₃ MAb to LDL. D₆ binds to the amino
10 terminal half of Apo B-100 whereas HB₃CB₃ binds to the carboxy terminal end of B-100. Thus, both MAbs can bind simultaneously to the same LDL particle due to sufficient steric distance between their corresponding epitopes. The Pan B (D₆) MAb,
15 biotinylated at the Fc fragment as described above, was mixed with streptavidin-peroxidase (BRL, Bethesda, MD) to form antibody-peroxidase complex due to binding between the biotin on the antibody and the streptavidin conjugated to peroxidase. The
20 complex was dialyzed against PBS containing 25% sucrose (Sigma, St. Louis, MO). This peroxidase-tagged Pan B (D₆) MAb complex was still capable of recognizing and binding to LDL. PVDF strips were coated with anti-LDL antibody(HB₃CB₃) and blocked as
25 described above. Anti-LDL dipsticks were incubated with human serum samples for 2 minutes, washed under tap water, air dried for 2 minutes and incubated with biotinylated Pan B (D₆) MAb and streptavidin-peroxidase for 10 minutes. After the
30 incubation with the Pan B (D₆) MAb-peroxidase complex, the dipsticks were washed with tap water and incubated for 2 minutes with the chromogenic peroxidase substrate TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD). This was followed
35 by an additional 1-minute washing under tap water and drying at room temperature for 5 minutes. The Pan (D₆) MAb- peroxidase complex bound to LDL captured by the anti-LDL strip and converted TMB

substrate into a colored compound. The whole procedure was carried out at room temperature. The intensity of the blue-green color of dipsticks was proportional to the concentrations of

- 5 LDL-cholesterol in the respective serum samples as shown by the data in Table 6 below.

In Table 6, serum LDL-C was determined in all samples by direct LDL-C assay (Sigma, St. Louis, MO). The same serum samples were incubated with
10 anti-LDL dipsticks followed by incubation with D₆ MAb-peroxidase and staining with TMB substrate as described above. The color intensity was assessed visually (on an arbitrary scale of 1 to 15) by
15 three individuals (I, II and III) presented with the complete set of 16 dipsticks at the same time. The averaged score of the color intensity correlated significantly with LDL-C concentrations ($r=0.98$, $p<0.0001$).

Table 6: Correlation between the serum LDL-cholesterol concentration and the color intensity of HB_{CB} coated (anti-LDL) dipsticks stained with D₆ Mab- peroxidase-TMB system.

Sample number	Serum LDL-cholesterol (mg/dl)	I	II	III	Average color score
1	145	5	4	4	4.3
2	130	4	3	5	4.0
3	97	2	2	3	2.3
4	165	7	7	6	6.7
5	115	3	2	3	2.7
6	200	8	9	9	8.7
7	207	8	9	9	8.7
8	160	5	6	7	5.7
9	115	2	2	2	2.0
10	276	15	15	15	15.0
11	155	6	4	4	4.7
12	98	1	2	2	1.7
13	123	3	3	3	3.0
14	130	3	4	4	3.7
15	185	6	6	9	7.0
16	73	1	1	1	1.0

To summarize, the above experiments demonstrated that anti-LDL dipsticks allow for quantification of LDL by visualizing either lipids by staining with lipid stains or Apo-B by staining with a protein stain or chromogenic assay using a substrate such as TMB.

Example 8: Dipstick Method to Assay HDL by Staining of Apolipoprotein A-I.

Anti-HDL dipsticks coated with A1bD₅ MAb were used to adsorb HDL particles in human serum samples as described above. After washing, the dipsticks were incubated with the second MAb to Apo-I (A1bE₂) complexed with streptavidin peroxidase as described above. The A1bE₂-peroxidase complex bound to the HDL particles which were captured on the dipstick by the A1bD₅ MAb. After incubation with TMB, washing, and drying, the color intensity was proportional to the serum HDL cholesterol as shown by the data in Table 7 below.

In Table 7, serum HDL-cholesterol was determined in all samples by a commercial kit (Sigma, St. Louis, MO). The same serum samples were incubated with anti-HDL dipsticks followed by incubation with A1bE₂ MAb-peroxidase complex and staining with TMB as described above. The color intensity was assessed visually (on an arbitrary scale of 1 to 10) by three individuals (I, II and III) presented with the complete set of 9 dipsticks at the same time. The averaged score of the color intensity correlated significantly with HDL-C concentrations ($r=0.97$, $p<0.0001$)

Table 7: Correlation between the serum HDL-cholesterol concentration and the color intensity of AIBD₅ (anti-HDL) dipsticks stained with AIB₅, MAB-peroxidase-TMB system

Sample number	Serum LDL-cholesterol (mg/dl)	Dipstick color intensity			Average color score
		I	II	III	
1	65	10	10	10	10.0
2	53	6	7	6	6.3
3	58	6	7	7	6.7
4	48	6	5	5	5.3
5	40	5	4	5	4.7
6	60	8	8	8	8.0
7	28	1	1	1	1.0
8	37	4	4	4	4.0
9	39	4	4	4	4.0

Both Pan B (D₆) MAb- and A1bE₂ MAb-peroxidase complexes were stable in PBS containing 25% sucrose for at least 3 months at 4°C.

Example 9: Dipstick Method to Assay the LDL/HDL Ratio.

5 Anti-LDL and anti-HDL dipsticks, prepared as described above, were used to determine the relative ratio of LDL-Apo B to HDL-Apo A-I, that is, the LDL/HDL ratio. Small pieces (0.5 x 0.5 cm) of both anti-HDL and anti-LDL dipsticks were simultaneously incubated with the same sample of human serum, plasma, or whole blood for 2 minutes, washed under tap water, air dried for 2 minutes and incubated with an equimolar mixture of D₆ MAb- and A1bE₂ MAb-streptavidin-peroxidase complexes for 10 minutes to detect bound LDL and HDL, respectively. This was followed by washing under tap water, a 2-minute air drying, and a 2-minute incubation with the TMB substrate as described above. After an additional washing under tap water and air drying at room temperature (5 minutes) the color intensity on both dipsticks was compared visually. The serum, plasma or blood samples with known concentrations of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were analyzed by the above dipstick method. Sera with LDL-C concentrations between 110 mg/dl and 130 mg/dl and HDL-C concentrations between 40 and 55 mg/dl showed comparable color intensity on both dipsticks. Sera with LDL-C values higher than 140 mg/dl generally show more intense color on anti-LDL dipsticks. The only exceptions were the sera with HDL-C levels higher than 50 mg/dl. In these cases, anti-HDL dipsticks tended to be more intensely stained unless the LDL-C levels exceeded 160 mg/dl.

Virtually identical results were obtained when anti-HDL and anti-LDL dipsticks were used separately. In these experiments each serum with

previously determined HDL-C and LDL-C was separately incubated with anti-HDL and anti-LDL dipsticks and stained with AIB₂ and D₆ MAb-peroxidase complexes, respectively. The agreement between these two types of experiments demonstrates that even in case of simultaneous incubation of anti-HDL and anti-LDL dipsticks with the same serum, plasma or blood sample followed by the simultaneous incubation of both dipsticks with the mixture of AIB₂ MAb- and D₆ MAB-peroxidase, the reactions between lipoproteins and corresponding MABs coated on the dipsticks remain specific. HDL particles always bind to the AIB₂ MAB coating the anti-HDL dipstick and the AIB₂ MAB-peroxidase complex binds to the HDL-Apo A-I captured by the anti-HDL dipstick. The same is true for LDL particles which bind exclusively to the HB₃CB₃ MAB coating the anti-LDL dipstick and react with the D₆ MAB-peroxidase complex.

These studies demonstrate that the dipstick methodology provides a quick and simultaneous determination of relative quantities of HDL and LDL in serum, plasma, or whole blood samples. To determine the LDL/HDL ratio in an unknown blood sample, the color intensities on the HDL and LDL dipsticks, which were incubated with the unknown sample, are compared to a set of printed color standards derived from blood samples with known LDL/HDL ratios.

Example 10: Dipstick Method to Assay LPA-I/LPA-I:A-II Ratio.

To determine the LP A-I/LP A-I:A-II ratio, two dipsticks were used. One of them was coated with AIB₂ MAB (anti-Apo A-I) and the other with CdB₃ MAB (anti- Apo A-II). In addition to these two MABs, a third MAB, AIB₂ (anti-Apo A-I) was also used. AIB₂ was biotinylated at the Fc portion of IgG molecule and complexed with streptavidin-peroxidase

as described above. Samples of EDTA treated whole blood, serum and plasma were simultaneously incubated with both anti-Apo A-I and anti-Apo A-II dipsticks for 2 minutes at room temperature.

- 5 Dipsticks were then washed under tap water, incubated with the AIB₂ MAb-peroxidase complex for 10 minutes, washed again, incubated with TMB substrate, washed and air dried as described above. Intensities of the blue-green color developed on
- 10 both dipsticks were compared visually. The dipstick coated with AIB₂ MAb (anti-Apo A-I) captured both LP A-I and LP A-I:A-II particles, and it was always more intensely stained relative to the CdB₂ (anti-Apo A-II) coated dipstick. The
- 15 latter dipstick captures only LP A-I:A-II subfraction which represents approximately 60% of all Apo A-I-containing particles (Koren, E. et al. Clin. Chem., 33:38-43 (1987)). However, there were clear differences between various blood samples.
- 20 For example, in males, the difference between AIB₂ (anti-Apo A-I) dipsticks and the CdB₂ (anti-Apo A-II) dipsticks, although present, were generally less noticeable due to somewhat weaker staining of the anti-Apo A-I dipsticks. In
- 25 females, AIB₂ dipsticks were usually more intensely stained relative to CdB₂ coated dipsticks, reflecting higher concentrations of Lp A-I in their blood (Koren, E. et al., Clin. Chem., 33:38-43 (1987)). In addition to these observations, there was a good
- 30 correlation between the relative color intensities of both dipsticks and their respective particles determined by the ELISA described by Koren, E. et al. Clin. Chem. 33:38-43 (1987), incorporated herein by reference. These experiments demonstrate
- 35 that the dipstick methodology can be successfully used for a quick determination of LP A-I/LP A-I:A-II ratio. To determine the LP A-I/LP A-I:AI

ratio in an unknown blood sample, the color intensities of the anti-Apo A-I and anti-Apo A-II dipsticks which were incubated with the unknown sample are compared to a set of printed color standards derived from dipsticks incubated with blood samples with known concentrations of LP A-I and LP A-I:A-II.

Example 11: Dipstick Method to Assay the Distribution of Apo C-III and Apo-E (C-III ratio and E ratio).

The "C-III Ratio" has been shown to be a reliable indicator of the progression of coronary artery disease (Alaupovic, P. and Blankenhorn, D.H. Klin. Wochenschr., 60:38-40 (1990); Blankenhorn, D.H. et al. Circulation, 81:470-478 (1990)). The current methodology for the C-III ratio is based on precipitation of all Apo B-containing lipoprotein particles with heparin and quantification of Apo C-III in both the heparin precipitate and heparin supernatant fraction. The Apo C-III in the heparin precipitate fraction represents Apo C-III associated with Apo B in VLDL and VLDL remnant particles. Apo C-III remaining in the supernatant fraction is associated with HDL particles. The C-III ratio is calculated by dividing the Apo C-III in the heparin supernatant by the Apo C-III in the heparin precipitate. A low C-III ratio is associated with progression of coronary disease.

The dipstick methodology described above was also used to determine the distribution of Apo C-III, that is, to obtain a C-III ratio. PVDF strips were coated with the Pan B (D₆) MAb, blocked and incubated with human serum plasma or whole blood as described above. This was followed by washing, an incubation with the XbA₁ (anti-Apo C-III) MAb-peroxidase complex, an additional washing, and an incubation with chromogenic TMB substrate as described above. The color developed

on the Pan B (D₆) MAb-coated dipsticks was proportional to the Apo C-III associated with Apo B. As described earlier, the Pan B (D₆) MAb binds all Apo B-containing particles, including LDL.

5 However, the amount of Apo C-III associated with Apo B in LDL is negligible. Therefore, the color intensity on the Pan B coated dipsticks reflected the amount of Apo C-III associated with VLDL and VLDL remnant particles. The anti-Apo A-I (or anti-
10 HDL) dipsticks coated with A1bD₅ MAb were also used in combination with the XbA₃ MAb-peroxidase complex. The color on these dipsticks was proportional to the amount of Apo C-III associated with Apo A-I in HDL particles.

15 A visual comparison of the Pan B and the anti-HDL dipsticks after incubation with the same serum sample and staining, allowed for an estimation of the C-III ratio. A serum with a high C-III ratio (as determined by assaying C-III in a
20 heparin supernatant and precipitate) showed relatively strong color on the anti-HDL dipstick and only a faint color on the Pan B (D₆) MAb dipstick. A serum, which was previously shown to have a low C-III ratio, showed more intense color
25 on the Pan B (D₆) MAb dipstick and relatively weak color on the anti-HDL MAb dipstick. Identical results were obtained with whole blood.

Similar experiments were carried out to determine the Apo E ratio by the use of appropriate
30 dipsticks. As described above, the Apo E ratio is determined by dividing Apo E in heparin supernate with the Apo E in heparin precipitate. The Apo E ratio is analogous to the C-III ratio and reflects the quantity of VLDL and their remnants relative to
35 the HDL particles. To determine the Apo E ratio, the Pan B and anti-HDL dipsticks (coated with D₆ and A1bD₅ MAbs, respectively) were incubated with human

serum, plasma or whole blood for 2 minutes, washed under tap water, air dried for 2 minutes and incubated with an equimolar mixture of two anti-Apo E MAb (each complexed with

5 streptavidin-peroxidase) for 10 minutes. This was followed by washing and incubation with TMB substrate as described above. Two anti-Apo E MAb (Efb₁ and Efd₃) were separately biotinylated at their Fc fragments and complexed with the strept-

10 avidin-peroxidase as described above. Since Efb₁ MAb binds predominantly to Apo E associated with VLDL, whereas Efd₃ preferentially binds Apo E on HDL particles, an equimolar mixture of Efb₁ and Efd₃-peroxidase complexes was used for incubation with

15 the Pan B and anti-HDL dipsticks. This mixture was used in place of a single MAb with equal binding to all Apo E-containing lipoproteins. Nevertheless, determination of the Apo E ratio with the above dipsticks is quite similar to the Apo C-III ratio.

20 The sera with low Apo E ratio determined by the heparin precipitation method gave a relatively weak staining on anti-HDL dipsticks, reflecting a low concentration of Apo E associated with Apo A-I in HDL particles, and an intense staining on the Pan B

25 dipsticks due to the high concentration of Apo E associated with Apo B. The sera with high Apo E ratio gave an inverse pattern (strong staining of anti-HDL and weaker staining of Pan B dipsticks).

These experiments demonstrate that a dipstick

30 technique using described combinations of MAb to apolipoproteins A-I, B, C-III and E provides a quick (approximately 30 minutes) estimation of the C-III and E ratios in human serum, plasma and whole blood, or other biological samples. The

35 conventional determinations of these ratios cannot be done with whole blood and take 12-24 hours

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(Alaupovic, P. Can. J. Biochem., 59:565-579 (1981)).

Example 12: Production of Recombinant Anti-LDL Antibody.

5 Murine hybridoma cells producing anti-LDL HB₃CB₃ MAb were used to produce recombinant anti-LDL using a commercially available recombinant phage antibody system (RPAS, Pharmacia Biotech Inc., Piscataway, NJ). Briefly, mRNA was isolated from
10 HB₃CB₃ producing hybridoma cells, followed by synthesis of cDNA encoding the variable regions of both heavy and light chains of the HB₃CB₃ MAb. The heavy and light chain encoding cDNAs were amplified
15 in two separate PCRs using two sets of primers, specific for each chain. The amplified heavy and light chain cDNA fragments were then purified using agarose gel electrophoresis and assembled into a single recombinant DNA fragment using a DNA linker fragment (Pharmacia Biotech Inc., Piscataway, NJ).
20 The resulting recombinant DNA molecule encodes a single chain polypeptide, called a single chain Fv fragment (ScFv), which binds the same epitope as the original MAb.

The recombinant DNA fragment was approximately
25 700 base pairs in length. The assembled ScFv DNA was amplified with a set of oligonucleotide primers that introduced the *Sfi*I and *Not*I restriction sites. This recombinant DNA fragment was further purified and sequentially digested with *Sfi*I and
30 *Not*I to generate cohesive ends for ligation into the phage plasmid (phagemid) pCANTAB 5 (Pharmacia Biotech Inc., Piscataway, NJ) cloning vector. The inserted recombinant DNA encoding the ScFv was fused with the 5' end of the gene coding the g3p
35 minor coat protein located at the tip of the phage. The ligated phagemid vector containing the inserted DNA, was introduced into competent *E. coli* TG 1 cells.

Phagemid-containing bacterial colonies were infected with M13 KO7 helper phage to yield recombinant phage which display ScFv antibodies. At this stage, recombinant anti-LDL ScFv antibody

5 is expressed on the tip of the phage as a fusion product between the antigen-binding site of HB₃CB₃ MAb and the M13 g3p minor coat protein. Phage, containing phage-displayed ScFv antibodies capable of binding LDL, were selected by panning in

10 LDL-coated cell culture flasks. The panning and reinfection of *E. coli* TG 1 cells was repeated several times until phage-displayed ScFv antibodies of high affinity were obtained. The LDL binding affinity of the ScFv antibodies was determined

15 using an ELISA method. The wells of microtiter ELISA plates were coated with serial dilutions (80 to 2.5 µg/ml) of LDL and blocked as described above. Phage displaying anti-LDL ScFv antibodies were pipetted into duplicate wells and allowed to

20 bind for 3 hours at room temperature (approximately 25°C). After washing, the peroxidase-labelled sheep antibody directed against the M13 g8p major coat protein was added to detect the presence of recombinant phage antibodies bound to LDL. After

25 washing, a peroxidase chromogenic substrate (ABTS, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added and the resulting color intensity measured by the use of an ELISA plate reader (MR 580, Dynatech Chantilly, VA). The serial dilutions

30 of LDL gave rise to a binding curve for each recombinant phage anti-LDL. The slopes of binding curves were compared to the slope of the native anti-LDL HB₃CB₃ MAb which was used on each plate as a positive control. Out of 35 phage ScFv antibodies,

35 several showed affinities comparable to HB₃CB₃ MAb based on the slopes of binding curves as shown in Table 8.

In Table 8, ELISA plates were coated with LDL using concentrations indicated in the left column. LDL coated wells were incubated (in duplicates) with HB₃cB₃ MAb (2 µg/ml) and RcB₃M₁D₄ recombinant phage antibody as described above. Detection of bound antibodies was carried out using the respective peroxidase labeled conjugates as described above. The numbers represent average optical density readings values from two separate experiments.

Table 8: Binding of HB₃cB₃ MAb (anti-LDL) and RcB₃M₁D₄ recombinant phage antibody to LDL.

LDL concentration µg protein/ml	HB ₃ cB ₃ monoclonal antibody	RcB ₃ M ₁ D ₄ recombinant antibody	Phage
80	0.802	0.675	
40	0.497	0.406	
20	0.263	0.211	
10	0.115	0.098	
5	0.060	0.047	
2.5	0.042	0.036	

ScFv phage antibody with the highest affinity (RcB₃M₁D₄) was placed on deposit at the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852) under the ATCC designation number 69602.

Example 13: Use of Recombinant Phage Anti-LDL ScFv Antibodies to Detect LDL.

Intact phage displaying the recombinant anti-LDL ScFv antibody RcB₃M₁D₄ were used in both ELISA and dipstick methods to detect LDL. ELISA microtiter plates were coated with a sheep antibody to M13 g8p coat protein (Pharmacia Biotech Inc., Piscataway, NJ) and blocked with a 0.1% nonfat milk

proteins as described above. After washing, the recombinant phage anti-LDL ScFv antibodies were added to the plate and allowed to bind to the anti-M13 g8p antibody overnight at 4°C. Because the anti-LDL ScFv antibody is expressed on the tip of the phage as a protein fused to the minor coat g3p protein, the anti-LDL binding site is free to bind LDL. Unbound recombinant phages were washed off and the wells incubated with dilutions of LDL (3 hours at room temperature). The unbound LDL was then washed away, and the Pan B (D₆) MAb antibody-peroxidase complex was added and incubated as described above. Unbound Pan B (D₆) MAb was washed away and the chromogenic peroxidase substrate (ABTS) was added. The color intensity in each well was read using an ELISA plate reader. The color intensity correlated with the concentration of LDL used in each well of the plates. The negative control wells coated with the native M13 phage showed no color at all.

The phage anti-LDL ScFv antibody RCB₃M₁D₄ was also used to explore its suitability for the dipsticks. PVDF strips were sequentially coated with the anti-g8p antibody and blocked with 2% nonfat milk proteins as described above. This was followed by three sequential 24-hour incubations of the strips in a solution of RCB₃M₁D₄ phage antibody at 4°C. After washing in PBS, strips were incubated with LDL dilutions followed by an incubation with the Pan B (D₆) MAb-peroxidase complex, washing, and incubation in TMB chromogenic substrate as described above for the HB₃CB₃ MAb dipsticks. The intensity of color on the phage anti-LDL ScFv antibody-coated dipsticks was proportional to the concentration of LDL. The experiments with the phage anti-LDL demonstrate that the recombinant anti-LDL ScFv antibodies are

capable of binding to LDL under the conditions used in described ELISA as well as dipstick methods. Thus, anti-LDL ScFv antibodies made free of the phage components (Hoogenboom, H.R. et al. Nucl. Acid. Res. 19:4133-4137 (1991)) are likewise suitable, for use in methods and compositions such as ELISA and dipstick methodologies described above.

The rest of the hybridomas described above, which which produce the corresponding MAbs: AIbD₅, AIbE₂, CdB₅, XbA₃, EfB₁, EfD₃, are also useful to create a library of corresponding recombinant antibodies. This approach offers several important advantages. ScFv-encoding DNA recombinant molecules have also been produced from cDNA of the AIbD₅ and D₆ hybridomas and can be inserted into pCANTAB5 for making recombinant antigen-specific antibodies such as the RcB₃M₁D₄ anti-LDL recombinant phage antibody described above.

Modifications and variations of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

adding to the sample antibody molecules immunoreactive with a specific lipoprotein or apolipoprotein, wherein the antibody specifically binds to a stable, conformation independent epitope which is uninfluenced by the lipid content;

determining the amount of lipoprotein, apolipoprotein, or lipid associated with a lipoprotein bound by the immobilized antibody molecules.

3. The method of claim 2 wherein the antibody is selected from the group consisting of monoclonal antibodies, recombinant antibodies, and antibody fragments.

5. The method of claim 3 wherein the antibody is a recombinant anti-LDL RCB₃M₁D₄ ATCC designation number 69602.

6. The method of claim 1 wherein the antibodies are immobilized onto a solid phase material, further comprising separating the solid phase material containing the immobilized antibody molecules from the biological sample.

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13. The method of claim 1 further comprising:
mixing an antibody immunoreactive with a
specific apolipoprotein into the sample;
allowing the antibody to bind to the
apolipoprotein in the sample,
adding to the mixture a second antibody
immunoreactive with a second, distinct epitope of
the apolipoprotein,
allowing the second immobilized antibody to
bind to the apolipoprotein,
detecting the presence of the
apolipoprotein bound by both antibodies, and

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determining the amount of apolipoprotein bound by both antibodies.

14. The method of claim 13 wherein the apolipoprotein is apolipoprotein Apo B-100.

Sub
B'

15. The method of claim 13 for determining the relative ratio of VLDL to HDL comprising determining the amount of VLDL in a sample based on the amount of Apo C-III present in the VLDL in the sample by

providing Pan B antibody which is characterized by an equal binding and high affinity for all Apo B-containing lipoproteins in human plasma,

providing soluble antibody immunoreactive with Apo C-III having binding affinity and specificity similar to XbA₃,

mixing the soluble antibody reactive with Apo C-III with the biological sample to form complexes between the soluble antibody and the Apo C-III containing lipoprotein particles,

adding the immobilized Pan B antibody to the biological sample, and

determining the amount of Apo C-III associated with Apo B, which is the amount of Apo C-III present in VLDL in the sample; and

determining the amount of HDL in a sample based on the amount of Apo C-III present in the HDL in the sample by

providing Apo A-I antibody immunoreactive specifically with Apo A-I having a binding affinity and specificity similar to A1bD₃ and A1bE₂,

providing soluble antibody immunoreactive with Apo C-III having binding affinity and specificity similar to XbA₃,

mixing the soluble antibody reactive with Apo C-III with the biological sample to form complexes between the soluble antibody and the Apo C-III containing lipoprotein particles,

Sub B' 15. The method of claim 13 for determining the relative ratio of VLDL to HDL comprising determining the amount of VLDL in a sample based on the amount of Apo C-III present in the VLDL in the sample by

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immersing the immobilized anti-Apo A-I antibody into the biological sample, and

determining the amount of Apo C-III associated with Apo A-I, which is the amount of Apo C-III present in HDL in the sample.

16. The method of claim 13 for determining the relative ratio of VLDL to HDL comprising

determining the amount of VLDL in a sample based on the amount of Apo E present in the VLDL in the sample by

providing Pan B antibody which is characterized by an equal binding and high affinity for all Apo B-containing lipoproteins in human plasma,

providing a mixture of soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to EfB₁ which binds to Apo E associated predominantly with VLDL and soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to EfD₁ which binds to Apo E associated predominantly with HDL,

adding the mixture of soluble antibodies reactive with Apo E to the biological sample to form complexes between the soluble antibodies and Apo E containing particles,

immersing the immobilized Pan B antibody into the biological sample, and

determining the amount of Apo E associated with Apo B which is the Apo E present predominantly in VLDL in the sample; and

determining the amount of HDL in a sample based on the amount of Apo E present in the HDL in the sample by

providing Apo A-I antibody immunoreactive specifically with Apo A-I having a binding affinity and specificity similar to A1bD₅,

providing a mixture of soluble antibody immunoreactive with Apo E having binding affinity

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and specificity similar to EfB₁, which binds to Apo E predominantly associated with VLDL, and soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to EfD₃, which binds to Apo E predominantly associated with HDL,

adding the mixture of soluble antibodies reactive with Apo E to the biological sample to form complexes between the soluble antibodies and Apo E containing particles, and

determining the amount of Apo E associated with Apo A-I, which is the amount of Apo E present in HDL in the sample.

17. The method of claim 13 for determining the relative ratio of LPA-I and LPA-II lipoprotein particles comprising

providing anti-Apo A-I antibody immunoreactive specifically with Apo A-I having a binding affinity and specificity similar to AIBD₅;

providing anti-Apo A-II antibody immunoreactive specifically with Apo A-II having a binding affinity and specificity similar to CdB₅;

mixing the soluble anti-Apo A-I antibody having a binding affinity and specificity similar to AIBD₅ to form complexes with both LPA-I and LPA-I:AI;II;


immersing the anti-Apo A-I antibody into the biological sample and determining the quantity of Apo A-I associated with both LPA-I and LPA-II lipoprotein particles;

immersing the anti-Apo A-II antibody into the biological sample and determining the quantity of Apo A-I associated with the LPA-I:AI:II.

18. A composition for determining the concentration of a lipoprotein, apolipoprotein, or lipid associated with a specific lipoprotein in a biological sample comprising:


a solid phase material having immobilized thereon antibody molecules specifically

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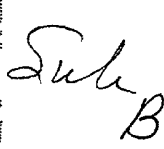


immunoreactive with a specific lipoprotein or apolipoprotein, wherein the antibody specifically binds to a stable, conformation independent epitope which is uninfluenced by the lipid content.

~~19. The composition of claim 18 further comprising a solid support to which the solid phase material is attached to form a dipstick.~~


Sub a2 

~~20. The composition of claim 18 wherein the antibody is selected from the group consisting of monoclonal antibodies, recombinant antibodies, and antibody fragments.~~

Sub B2 


~~21. The composition of claim 18 wherein the antibody is the anti-LDL monoclonal antibody produced by the hybridoma cell line HB₃CB₃ ATCC designation number HB 11612.~~

~~22. The composition of claim 18 wherein the antibody is a recombinant anti-LDL RCB₃M₁D₄ ATCC designation number 69602.~~

Sub B2 

~~23. The composition of claim 18 further comprising a solution containing molecules of a second soluble antibody immunoreactive with a second distinct epitope of the lipoprotein or apolipoprotein which is immunoreactive with the antibody molecules immobilized on the solid phase material.~~

~~24. The composition of claim 18 wherein the antibody molecules are immobilized to the solid phase material using avidin-biotin complexes.~~

Sub a4 

~~25. The composition of claim 19 further comprising at least one internal standard comprising a known amount of a particular lipoprotein, lipoprotein lipid, or apolipoprotein immobilized on the solid phase material.~~

~~26. The composition of claim 18 wherein the solid phase material is selected from the group consisting of nitrocellulose, polyvinylidene~~

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difluoride, partially acid-hydrolyzed nylon,
~~polystyrene, polypropylene, and paper~~

27. The composition of claim 18 wherein the apolipoprotein is selected from the group consisting of Apo A-I, Apo A-II, Apo B, Apo C-III, and Apo E.

Sub B3
~~28. The composition of claim 18 for determining~~
the relative ratio of VLDL to HDL comprising immobilized Pan B antibody which is characterized by an equal binding and high affinity for all Apo B-containing lipoproteins in human plasma,

soluble antibody immunoreactive with Apo C-III having binding affinity and specificity similar to XbA₃,

immobilized Apo A-I antibody immunoreactive specifically with Apo A-I having a binding affinity and specificity similar to AIBD₅ and AIB₂, and

soluble antibody immunoreactive with Apo C-III having binding affinity and specificity similar to XbA₃.

29. The composition of claim 18 for determining the relative ratio of VLDL to HDL comprising

immobilized Pan B antibody which is characterized by an equal binding and high affinity for all Apo B-containing lipoproteins in human plasma,

a mixture of soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to EFB₁ which predominantly binds to Apo E associated with VLDL and soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to EFD₃ which predominantly binds to Apo E in HDL,

immobilized Apo A-I antibody immunoreactive specifically with Apo A-I having a binding affinity and specificity similar to AIBD₅, and

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a mixture of soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to Efb₁ which binds to Apo E predominantly associated with VLDL and soluble antibody immunoreactive with Apo E having binding affinity and specificity similar to Efd₃ which predominantly binds to Apo E in HDL.

30. The composition of claim 18 for determining the relative ratio of LPA-I and LPA-II lipoprotein particles comprising

immobilized Apo-A-I antibody which binds Apo A-I lipoproteins in human plasma having a binding affinity and specificity with Apo AIbD₅; and

immobilized Apo A-II antibody immunoreactive specifically with Apo A-II having a binding affinity and specificity similar to CdB₅.

~~31. A method for making a composition comprising~~

immobilizing on a solid phase material antibody molecules immunoreactive with a specific lipoprotein or apolipoprotein, wherein the antibody molecules are selected from the group consisting of monoclonal antibodies, recombinant antibodies, and fragments thereof, and wherein the antibody has a binding affinity of at least 10⁹ for a stable, conformation independent epitope which is uninfluenced by the lipid content.

32. A method for making a composition for determining the concentration of a specific lipoprotein, an apolipoprotein, or lipid associated with a specific lipoprotein, in a biological sample comprising

immobilizing on a solid phase material antibody molecules immunoreactive with a specific lipoprotein or apolipoprotein, wherein the antibody molecules are selected from the group consisting of monoclonal antibodies, recombinant antibodies, and

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fragments thereof, and wherein the antibody specifically binds to a stable, conformation independent epitope which is uninfluenced by the lipid content.

33. The method according to claim 32 wherein the antibody molecule is specifically immunoreactive with LDL.

34. The method of claim 32 wherein the apolipoprotein is selected from the group consisting of Apo A-I, Apo A-II, Apo B, Apo C-III, and Apo E.

35. An antibody molecule specifically immunoreactive with LDL that does not significantly cross-react with other lipoproteins in whole blood, blood plasma or blood serum, wherein the molecule is selected from the group consisting of monoclonal antibodies, recombinant antibodies, and fragments thereof and wherein the antibody specifically binds to a stable, conformation independent epitope which is uninfluenced by the lipid content.

36. The antibody molecule of claim 35 wherein the antibody is the anti-LDL monoclonal antibody produced by the hybridoma cell line HB₃CB₃, ATCC designation number HB 11612.

37. The antibody molecule of claim 35 wherein the antibody is a recombinant anti-LDL RCB₃M₁D₄, ATCC designation number 69602.

38. The antibody molecule of claim 35 immobilized to a solid support.

39. The antibody molecule of claim 38 wherein the support is a resin for purification of apolipoprotein, lipoprotein, or lipid associated therewith.

40. A method for purifying an apolipoprotein comprising

reacting a solution containing apolipoprotein with an immobilized antibody selected from the

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group consisting of the anti-LDL monoclonal antibody produced by the hybridoma cell line HB₃CB₃ ATCC designation number HB 11612 and the anti-LDL RCB₃M₁D₄ recombinant antibody ATCC designation number 69602.

41. The method of claim 12 wherein binding of the second antibody forms a precipitate of the antigen and both bound antibodies which can be detected in solution.

Sub B4
42. The method of claim 1 for determining the relative ratio of LDL to HDL comprising
adding to the sample antibody molecules immunoreactive with low density lipoprotein and not cross-reactive with high density lipoprotein and determining the amount of low density lipoprotein;
adding to the sample antibody molecules immunoreactive with high density lipoprotein and not cross-reactive with low density lipoprotein and determining the amount of high density lipoprotein;
and
determining the ratio of the amount of low density lipoprotein with the amount of high density lipoprotein.

43. The method of claim 41 wherein the anti-low density lipoprotein antibody is selected from the group consisting HB₃CB₃ and recombinant RCB₃M₁D₄ antibodies.

44. The method of claim 43 wherein the method comprises

(a) determining the amount of low density lipoprotein in a sample by
providing immobilized anti-LDL antibodies,
providing soluble labelled anti-ApoB antibodies,
mixing the soluble anti-ApoB antibody in the biological sample to form complexes between the soluble antibody and the ApoB containing lipoprotein particles,

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determining the amount of ApoB captured by the immobilized antibody to calculate the amount of LDL,

(b) determining the amount of high density lipoprotein in a sample by

providing immobilized anti-ApoA-I antibody having a binding affinity and specificity similar to AIBD₅,

providing soluble labelled antibody having a binding affinity and specificity similar to AIBD₂,

mixing the soluble antibody reactive with ApoA-I in the biological sample to form complexes between the soluble antibody and the ApoA-I in lipoprotein particles,

determining the amount of ApoA-I captured by the immobilized antibody and calculating the amount of HDL from the amount of ApoA-I, and

(c) calculating the ratio of LDL to HDL.

~~add B5~~
~~add E1~~

Amended



[11] Patent Number: 6,107,045

[45] **Date of Patent:** Aug. 22, 2000

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Primary Examiner—Patricia A. Duffy
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ABSTRACT

Compositions and methods using antibodies which are immunoreactive with specific apolipoproteins to determine the concentrations of lipoproteins such as HDL and LDL, and/or apolipoproteins in human blood, serum or plasma sample, are described. Monoclonal antibodies (MAbs) are described that specifically bind to epitopes present in apolipoproteins and lipoproteins, enabling rapid and reliable determinations of levels of specific blood lipoprotein and/or apolipoprotein levels, including Apo B-100, Apo A-I, Apo A-II, Apo C-III, and Apo E, and thereby determination of relative ratios of HDL and LDL and Lp(a) and Lp(a). In a preferred embodiment, the compositions are strips of a solid phase material coated with one or more of the antibodies and are referred to herein as "dipsticks". The dipsticks specifically bind a lipoprotein or apolipoprotein when dipped into a protein sample. The amount of lipid associated with a bound lipoprotein or the amount of apolipoprotein bound on the dipstick is quantitated using an appropriate method, for example, by staining with a lipid stain or reaction with a second labelled antibody. The intensity of the stain on the dipstick is proportional to the concentration of the lipoprotein lipid or apolipoprotein circulating in the blood and can be quantitated by comparison with standards containing known amounts of lipid.

31 Claims, No Drawings

Applicant or Patentee: Eugen Koren and Mirna Koscec

Serial or Patent No.:

Attorney's
Docket No:
OMRF143 CIP

Filed or Issued: Herewith

For: Antibodies to Lipoproteins and Apolipoproteins and
Methods of Use Thereof

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
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I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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William G. Thurman 12-13-96
SIGNATURE DATE

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(Includes Reference to PCT International Applications)

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OMRF143 CIP

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Antibodies to Lipoproteins and Apolipoproteins and Methods of Use Thereof

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. _____

on _____,

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

Number PCT/US95/08331

on 30 June 1995,

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

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PCT	PCT/US95/08331	30 June 1995	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
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08/268,809	30 June 1994		X	
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

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